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No. 39.

STUDIES ON THE OXIDISING ACTION OF SELENIOUS ACID.

I. ORGANIC SULPHUR COMPOUNDS.

By A. E. A. WERNER.

[Read March 25. Published separately April 30, 1941.]

It has long been known that selenious acid and selenites are readily reduced to selenium by a large variety of chemical compounds, and recently Riley and his co-workers (1) in a series of papers have demonstrated the specific oxidising action of selenious acid towards a number of organic compounds, including aldehydes, ketones, alcohols, unsaturated hydrocarbons, and esters. Further work by Mel'nikov (2), who has shown that mercaptans can be reduced to disulphides, and a recent test for the detection of selenites, devised by Deniges (3), which depends on the production of a red precipitate of selenium by the addition of thiourea to the acidified solution of the selenite, has led to the present investigation of the oxidising action of selenious acid on a variety of organic compounds containing sulphur, including thiourea and its derivatives, thioamides, thioacids, mercaptans, and thiophenols. All these compounds either contain the easily oxidisable –SH group present in the molecule, or can give rise to it by a simple intramolecular change, so that the oxidising action of the selenious acid can be represented by the following general equation:—

$$4\ R\text{-}SH + H_2SeO_3 = 2\ R\text{-}S\ S\text{-}R + Se + 3\ H_2O$$

This reaction can be used as a specific test for the detection of the presence of the –SH group in organic sulphur compounds, because a red precipitate of selenium is always produced if the experimental conditions are suitably chosen, as will be discussed later. Furthermore, the different experimental conditions under which the reaction can be carried out provide valuable information concerning the molecular structure of the compounds under investigation.

The actual results obtained are best considered under the following headings:—

A. *Thiourea and Derivatives.* When selenious acid is added to an aqueous solution of thiourea, there is an immediate precipitate of selenium; in fact, the test devised by Deniges (*loc. cit.*) for the detection of selenites can serve equally well as a sensitive test for thiourea, since a distinct reddish turbidity due to colloidal selenium is produced with one part of thiourea in 50,000 parts of water. The author (4) has also evolved a method for the quantitative estimation of thiourea based on this reaction.

It is now found that all mono-, di-, and tri-substituted derivatives of thiourea react with selenious acid, producing either a *red* precipitate of selenium or an *orange* precipitate consisting of selenium contaminated with sulphur. It was

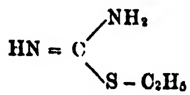
also found that the velocity of the reaction varies with the different derivatives, being instantaneous in the case of the parent thiourea, and decreasing progressively as one precedes from mono- to tri-substituted derivatives.

The essential results obtained may be summarised in the following table:—

TABLE. 1.—Reaction of substituted Thioureas with Selenious acid.

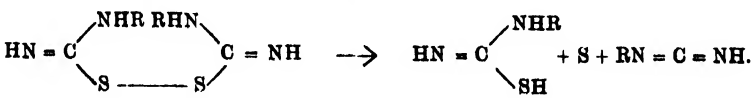
Substance tested.	Nature of Reaction.
Methyl thiourea ... ... Allyl thiourea	Immediate red ppt. of selenium.
Phenyl thiourea	Immediate orange ppt. of selenium and sulphur.
Sym-Diethyl thiourea ...	Slow reaction. Orange ppt.
Trimethyl thiourea ... ... Diethylbenzyl thiourea ... Dimethylphenyl thiourea ...	Very slow reaction. Orange ppt. of selenium and sulphur.

In the case of tetrasubstituted derivatives of thiourea or substituted isothioureas, e.g., ethyl isothiourea of the formula



no reaction with selenious acid can be observed.

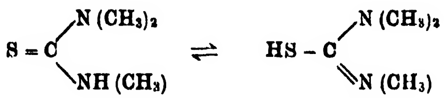
The sulphur with which the selenium precipitate is in many cases contaminated is derived from the decomposition of the resulting formamidine-disulphide according to the following equation:—



The extent to which this decomposition takes place depends, firstly, upon the nature of the substituent groups present in the thiourea molecule, and, secondly, upon the degree of acidity of the solution; the more strongly acid the solution the less is the amount of this decomposition. In the presence of excess strong mineral acids the formamidine-disulphides are comparatively stable, but decompose when these solutions are diluted with water. In agreement with this fact it was found, in the case of those substituted thioureas which gave an *orange* precipitate in weakly acid solution, that if the reaction were carried out in a solution of concentrated hydrochloric acid a deep *red* precipitate of selenium was rapidly formed quite free from any sulphur. The stability of substituted formamidine-disulphides in the presence of strong mineral acid is also indicated by the work of Fichter and Braun (5), who subjected various substituted thioureas to electrolytic oxidation, and succeeded in isolating the resulting formamidine-disulphides as salts with perchloric acid.

In the present case formamidine-disulphides are in fact the main product of the oxidising action of selenious acid on thioureas, as can readily be shown by the isolation from the filtrate of the insoluble salts formed on the addition of a saturated solution of picric acid.

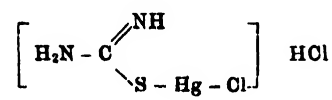
The results presented in Table 1 are best interpreted on the assumption that reduction of the selenious acid occurs provided there is present in the substituted thiourea molecule a free hydrogen atom which can migrate from nitrogen to sulphur, thus enabling the iso-thiourea structure containing the vulnerable –SH group to be attained. Thus, in the case of trimethylthiourea, for example, the following equilibrium is possible between the thiocarbamidic structure (A) and the iso-thiourea structure (B):—



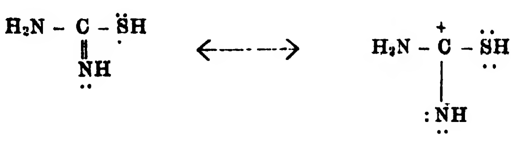
(A) (B)

The molecular form (B) reacts with selenious acid as rapidly as it is formed, so that the velocity of the reaction is governed by the extent to which the isomeric change A → B takes place. This change in turn is influenced greatly by the nature of the solvent, being favoured by a moderate increase in acidity.[1]

The fact that thiourea and its derivatives can exist in the iso-thiourea form is well known from the work of Werner (6) on the action of nitrous acid on thiourea, and also from the results of Ray (7) on the reaction between thiourea and mercuric chloride, in which a compound of the formula



is formed. Recently Clow (8) has produced evidence on a purely physical basis, namely, from measurements of the magnetic susceptibility, that thiourea (in the solid state) exists wholly as an amino-imino hybrid involving resonance between the two electronic structures,



whereas the derivatives of thiourea exist as a mixture of this amino-imino hybrid and the true thiocarbamidic form in varying proportions, depending upon the nature of the substituent group.

[1] The isomeric change also readily occurs in alkaline solution, but since only the free selenious acid acts as an oxidising agent there is no reaction when a selenite is added to an alkaline solution of thiourea.

Finally, this hypothesis that the isomeric change postulated above does actually occur in the reaction between selenious acid and the different substituted thioureas is confirmed by carrying out the reaction under experimental conditions of extreme acidity. For, in the presence of an excess of a very strong mineral acid, the thiourea molecule will be present as the salt of the formula

$$S = C\begin{matrix} \diagup \overset{+}{N}H_3 \\ \diagdown \overset{+}{N}H_3 \end{matrix}\ \bar{X}_2,$$

in which the vulnerable –SH group is not only absent but cannot arise by a simple isomeric change. In agreement with this view it was found that thiourea and its derivatives do not react with selenious acid when they are dissolved in such solvents as 50 per cent. sulphuric acid, 60 per cent. perchloric acid, or syrupy phosphoric acid. However, if these solutions are diluted with distilled water the reduction reaction starts, and the characteristic precipitate of selenium is formed. Furthermore, it is worthy of note that in the case of the parent thiourea itself the reduction reaction starts immediately after the addition of water, but in the case of the various derivatives of thiourea the precipitation of selenium takes place slowly at a rate determined by the velocity with which the isomeric change between the carbamidic and the iso-thiourea structures occurs.

The behaviour of the acyl derivatives of thiourea towards selenious acid was similar to that of the alkyl derivatives. Thus, monoacetyl and monobenzoyl thiourea both react very slowly in aqueous solution, or in the presence of a weak acid such as acetic acid, with the formation of an orange-yellow precipitate consisting of a mixture of selenium and sulphur; however, in the presence of a large excess of hydrochloric acid the reaction is not only exceedingly rapid, but also the precipitate formed is red in colour, and contains no sulphur. These results are best interpreted on the assumption that the monoacyl derivatives of thiourea are only converted very slowly in the absence of a strong mineral acid into the iso-thiourea form, and, secondly, that the resultant acyl formamidine-disulphide is very unstable in aqueous or weakly acid solution, and readily decomposes with the liberation of sulphur.

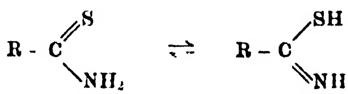
In the case of diacetyl thiourea there is no reaction with selenious acid in aqueous or weakly acidic solutions, but a very rapid reaction occurs if the selenious acid is added to the diacetyl thiourea dissolved in concentrated hydrochloric acid. This result is in agreement with the formula proposed by Werner (9) for diacetyl thiourea, namely,

$$HN = C\begin{matrix} \diagup NHCOCH_3 \\ \diagdown S - COCH_3 \end{matrix}\ .$$

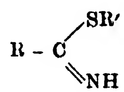
In the presence of a strong acid the acetyl group attached to the sulphur atom is readily removed by hydrolysis with the formation of monoacetyl thiourea,

which reacts as previously described. Finally, it may be noted that in the strongly acid solvents, such as 60 per cent. perchloric acid, these acyl derivatives behave similarly to the alkyl derivatives, and do not react with selenious acid until the solution is diluted with water.

B. *Thioamides.* Thioacetamide and thiobenzamide were chosen as typical representatives of this class of organic compounds; they were found to react immediately with selenious acid in aqueous solution or in glacial acetic acid, with the formation of an orange precipitate consisting of selenium mixed with a small amount of sulphur. This result is in agreement with the fact that thioamides can exist as an equilibrium mixture of the true thioamide form and the corresponding iso-thioamide form, namely,

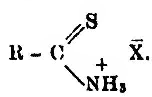


Evidence for this equilibrium has been produced from various sources, in particular the formation of S-ethers,



with alkyl halides.

In the presence of strong acid in excess the formation of the acidic iso-thioamide form is completely suppressed in favour of the basic thioamide form, which will be converted into the salt



This agrees with the experimental fact that in such solvents as 60 per cent. perchloric acid no reaction occurs on the addition of selenious acid, until the solution is diluted with water, when there is an immediate red precipitate of selenium.

C. *Thioalcohols and Thioacids.* The behaviour of these two classes of compounds is in direct contrast to that of the other sulphur compounds so far studied. In alcohol, dilute acid solution, or even when dissolved in glacial acetic acid, such compounds as ethyl mercaptan, thiophenol, thioglycollic acid, thiosalicylic acid, or cysteine, do not react with selenious acid; in the case of the thioacids a white crystalline mass precipitates out, which consists of a complex of the thioacid and selenious acid in molecular proportions. If this complex is dissolved in *concentrated* hydrochloric acid it decomposes with the precipitation of selenium, and formation of the corresponding disulphide acid. Furthermore, if these compounds are dissolved in very strongly acid solution, such as 60 per cent. perchloric acid or syrupy phosphoric acid, there is an immediate reaction on the addition of selenious acid, and a copious red precipitate of selenium is formed. This marked difference in behaviour may be due to the fact that in these thioalcohols and thioacids the sulphydryl group

is not so readily oxidised, as is the case with the thiourea and thioamide derivatives, unless the experimental conditions are especially favourable; apparently the most favourable conditions are those of extreme acidity. In this connection it is of interest to consider the reactivity of thioacids towards other oxidising agents. Thus, Schöberl and Krumey (10) have noted that the ease with which phosphotungstic acid is reduced by various thioacids depends upon the pH of the solution; also Schöberl and Rambacher (11) have found that the rate of splitting of the -S-S- linkage by sodium sulphite or potassium cyanide varies in different compounds, and depends on the pH of the solution. Furthermore, Schöberl (12) has found that *aa*-dimethyl- and diphenylthioglycollic acids were not oxidised by iodine in acid solution, whereas the primary thioglycollic acid and the secondary monomethyl-thioglycollic acid were both readily oxidised.

From the above results it is clear that the precipitation of selenium on the addition of selenious acid to sulphur-containing organic compounds under the appropriate experimental conditions might be used as a sensitive and specific test for the presence of the -SH group. In order to test this conclusion a large variety of other sulphur-containing organic compounds were treated with selenious acid, but in no case was there any precipitate of selenium formed. The compounds tested included:—(*a*) thioethers, e.g., diethyl- and diallyl-sulphide; (*b*) disulphides, e.g., dithiosalicylic acid; (*c*) cyclic sulphur compounds, e.g., thiophene, pseudothiohydantoin; (*d*) compounds containing the $> C = S$ group, e.g., isothiocyanates, diphenylthiohydantoin, tetramethylthiuramdisulphide.

It may be, therefore, taken as established that the precipitation of selenium on the addition of selenious acid constitutes a specific test for the presence of the sulphydryl group in organic sulphur compounds.

This communication is concerned mainly with the qualitative nature of the reaction between selenious acid and sulphur organic compounds, but it is hoped in a later communication to investigate this reaction in greater detail and on a quantitative basis.

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No. 40.

THE EFFECT OF WAVE-LENGTH ON THE RELATION BETWEEN THE INTENSITY OF ILLUMINATION AND THE CURRENT IN SELENIUM RECTIFIER PHOTO-CELLS.

By H. H. POOLE and W. R. G. ATKINS, F.R.S.

[Read March 25. Published separately July 14, 1941.]

In the course of standardising selenium rectifier photo-cells for use under water we found that the departure from linearity in the response of the cells in intense light was much greater in the deep red than in other parts of the spectrum (1). This curvature of the light/current characteristic increases with the resistance of the measuring circuit, and is generally attributed to the effect of the potential difference produced by the primary photo-electric transfer of electrons from the selenium to the overlying metal film which forms one electrode of the cell. This potential difference, which for a given illumination increases with the external resistance, drives some of the electrons back again, this leakage current increasing more rapidly than the voltage, with consequent reduction of the external current. Atkinson, Campbell, Palmer, and Winch (2) have pointed out that this cannot be a complete explanation, since their work shows, in confirmation of that of Büchmuller and König (3), that for moderate illuminations and low external resistances the effect may be reversed, the current increasing more rapidly than the voltage.

It seemed to us to be probable, however, that the greater fall in sensitivity with increasing current which we had observed with low-resistance circuits in red light was due to increased back-leakage, and we tentatively suggested that it might be due to the photo-conductivity of selenium, which has its maximum in the red part of the spectrum.

To examine the matter further a new photometer bench has been made, about 3·5 m. long and carrying at one end a 250-watt 220-volt projector lamp mounted in a dark lantern from which the light only emerged through a window of suitable size along the axis of the bench. The cells to be tested or compared were mounted on a carrier with a transverse slide, so that either could be brought into the exposing position behind the desired colour filter, the cell not in use being protected by an opaque screen. The cells were adjusted on the carrier so that the sensitive surface of whichever cell was exposed was in the same position relative to the carrier, and the distance of this surface from the plane of the lamp filament was set at some definite distance by sliding the carrier along the bench until it came against a suitable distance-piece interposed between it and a fixed block, which carried a large water cell interposing a thickness of 4·4 cm. of water in the path of the light beam. It was found that

with the carrier in contact with the block the distance of the cell surface from the plane of the filament was 13·7 cm. Refraction in the water cell reduces the effective optical distance to 12·45 cm. The cells tested were nearly 4 cm. in diameter, and the lamp filament approximately occupied a vertical rectangle about 1 2 cm. × 1·1 cm., so as an allowance for the finite sizes of source and cell 4·0 sq. cm. was added to the square of the optical distance when finding the lengths of the distance-pieces needed to give the desired relative illuminations. This was probably a slight over-correction (5), but as it only amounted to about 2 5 per cent. at the shortest distance any such error should not be serious. The distance-pieces were of such lengths that, when allowance was made for the water cell and for the sizes of source and photo-cell, each successive distance-piece or combination of distance-pieces reduced the light to 1/2. Thus if I were the illumination without any distance-piece—say about 15,000 lux (without filter)—the other values used were $I/2$, $I/4$, etc., down to $I/512$, with a final reduction factor of $I/\sqrt{2}$ to $I/725$, or about 20 lux. The filters used of course reduced the effects of these illuminations by different amounts. The long range and the large illuminations required rendered it impracticable to use the much more accurate additive method used for short ranges, such as 10 to 1, by Atkinson, Campbell, Palmer, and Winch.

The bench, which was carefully blackened, was set up in a dark room, and fixed and movable diaphragms prevented any appreciable amount of reflected or scattered light from reaching the cell. A shutter mounted close to the water cell protected the cell from light except during the few seconds while a reading was being taken, so that the effects of fatigue, or of the warming of the cell by the radiation, were minimized.

The lamp current was provided by a shunt-wound D.C. generator driven at very nearly constant speed by a three-phase motor, the voltage across the lamp being maintained as nearly as possible at 210 volts by hand regulation of a series rheostat. The photo-electric current was measured by the modified Campbell-Freeth balanced circuit which we have used at sea (4), except that a sensitive mirror galvanometer with suitable attenuator was used for balancing instead of the interrupter, amplifier, and telephone, and that an additional resistance of 1000 ohms could be inserted when required in series with the cell, thus changing the effective external resistance of the cell circuit from zero to 1000 ohms. The resistance controlling the balancing current ranged from 2,000 to 200,000 ohms, and the E.M.F. supplied by the potentiometer from a few millivolts to nearly 2 volts.

The first of a set of tests was made at the maximum distance, successive readings being taken with the shutter open and closed, so as to reduce greatly the small effect produced by any stray light scattered by the walls of the room. The tests were repeated with 1000 ohms in series with the cell, and then the second cell was brought into position and tested. The filter could then be changed for another filter or combination, thus giving a rapid comparison of the sensitivities for different spectral regions. The carrier was then moved up to the next position, and the operation repeated, and so on.

The results for two cells of different makes are plotted in figure 1. The relative intensities of illumination for a complete set of tests with a given filter are in the ratios $1/\sqrt{2}$, 1, 2, 4, . . . 512, hence we can find the relative values of the sensitivity (i.e. current/illumination) and express them as fractions of the sensitivity of the given cell and filter at the minimum illumination. As the only method of comparing the illuminations under different filters is by the resultant currents, which vary over a very wide range, the logarithm of this current is taken as abscissa. It may be noted that the Electrocell is considerably more sensitive than the Weston, as shown by the larger values of

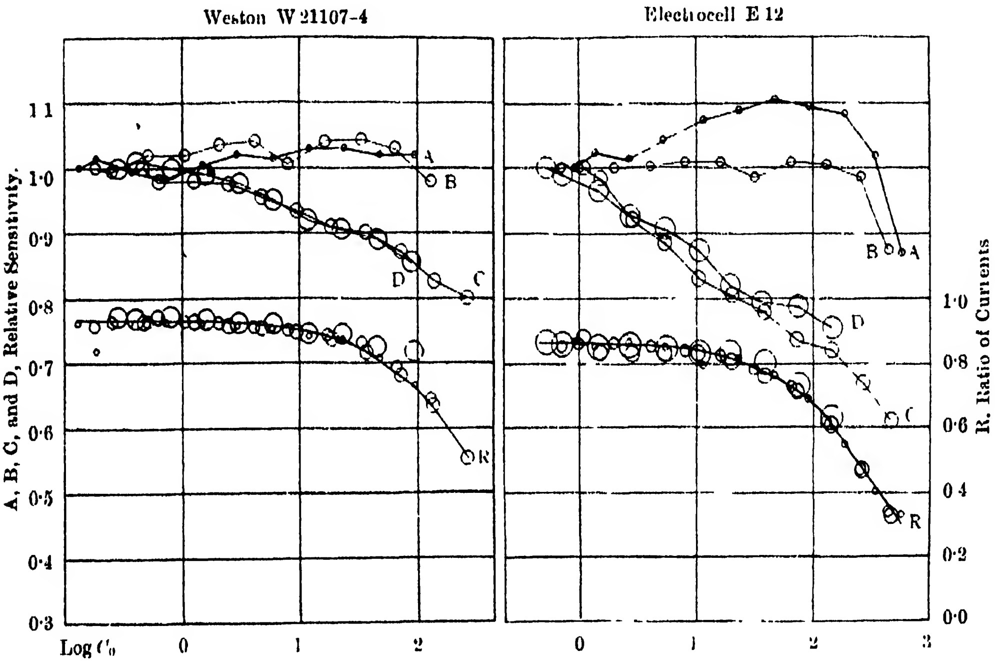

Fig. 1.—Variation with current of the sensitivity for zero external resistance and of the effective internal resistance of selenium cells when illuminated by light of various wave-lengths. *A*. Sensitivity with Schott BG 12 filter, about 3600 to 5000 Å. *B*. Sensitivity with Schott RG 1 and Corning Green filters, about 6000 to 6600 Å. *C*. Sensitivity with Schott RG 5 filter, over 6600 Å. *D*. Sensitivity with Schott RG 5 and Zeiss 966/S filters, over 7000 Å. *R*. Ratio of current through external resistance of 1000 ohms to current for same illumination through zero resistance. The same symbols are used for plotting the corresponding points as for the sensitivity curves. There is in this case, however, no significant difference between the results for different spectral bands. C_0. Current in microamperes for zero external resistance.

the current corresponding to the same bench settings. It is also relatively more blue-sensitive, as may be seen by comparing the maximum currents for curves *A*, *B*, and *C* for each cell. One reading in curve *B* is appreciably low for each cell. As these two readings were taken successively at the same bench setting it would seem that there must have been some small error in this, or that the voltage of the lamp may have fallen temporarily.

Both cells showed the initial increase of sensitivity with current through the zero-resistance circuit, as found by other workers, the Electrocell for blue light only, the Weston for both blue and orange-red. With deep red, however, the effect is quite different, and the comparatively sudden change near 6600 Å is very striking. There is no evidence that further increase of wave-length produces any further change, but curves *D* are not quite comparable with the others, as the small size of the only filter available having a cut-off about 7000 Å necessitated a modification in the arrangement, the filter being placed in a fixed position close to the water cell with the RG 5 filter in the standard position on the carrier to reduce the effect of any scattered light. This arrangement prevented the carrying out of measurements at the minimum distance attainable with the other filters.

The curves *R* show the ratios of the currents through 1000 ohm and zero-resistance circuits, and it is evident that apart from a few irregular points these fall fairly well on a smooth curve for each cell, regardless of what filter is used. Thus the change in curvature which sets in near 6600 Å is not due, as we had previously supposed, to an increase in the leakage current through the cell, as this would have been apparent as a fall in value of this ratio.

Figure 2 is plotted from results we obtained some years ago for a Weston cell, and it may be significant that the change of curvature which we have found

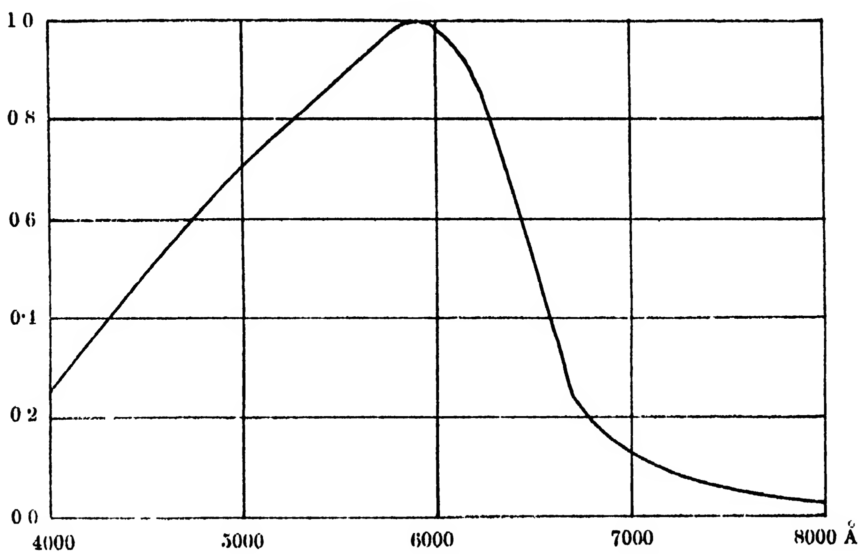

Fig. 2.—Relative spectral sensitivity of Weston selenium cell.

occurs about the region where the fall in sensitivity with increasing wave-length is at its steepest. It seems possible that a rectifier cell may possess more than one threshold, so that, starting with waves too long to energise any electrons in the selenium and enable them to cross the boundary into the metal, we may,

as the wave-length is reduced, affect successively electrons in different bands of energy levels. If we suppose that one such threshold occurs near 6600 Å there may be a considerable difference in the numbers of electrons responsive to light according as the wave-length is above or below that value. If we further suppose that the comparatively large currents generated in rectifier cells in bright light are sufficient to affect the equilibrium, and reduce appreciably the numbers of electrons in the upper energy levels, we would get a tendency to saturation in the current, which would be considerably more pronounced for wave-lengths above 6600 Å, as we observe. We would not expect to find any thresholds further down in the visible spectrum, since the sensitivity after reaching a maximum near 5900 Å falls again, this fall being possibly due to a combination of such causes as the reduction in the number of quanta per erg, a reduction with wave-length of the probability of an electron acquiring energy from a quantum, and/or a reduction in the percentage of the incident light which is transmitted by the overlying metal film. It is worth noting that the Electrocell, which is relatively more blue-sensitive than the Weston, and may have a maximum sensitivity further down the spectrum, shows curve *B* as intermediate between *A* and *C*, whereas for the Weston there is no significant difference between *A* and *B*. This may mean that for the Electrocell the threshold is rather below that for the Weston.

This attempt at an explanation does not account for the rise of sensitivity with illumination where the latter is moderate and of sufficiently short wave-length, and the external resistance is low. This effect, however, does not seem to be apparent in all cells, and may be due to some secondary cause. The increased fall in sensitivity with illumination in deep red light was found in every cell which we tested, namely, eight Electrocells and two Westons, so it seems likely to be a general property of selenium rectifier cells.

SUMMARY.

(1) Photometer bench tests of selenium rectifier cells in illuminations of different colours and covering a range of 725 to 1 for each colour show that the fall in sensitivity (i.e. current/illumination) with increasing illumination was greater for wave-lengths exceeding 6600 Å than for other spectral regions. The currents were measured by the Campbell-Freeth balanced circuit giving the effect of a galvanometer of zero resistance.

(2) When a resistance of 1000 ohms was included in the measuring circuit the resultant relative fall in the current for a given illumination was a function of the zero-resistance current only, and, for a given value of this, was independent of the colour of the light.

(3) It would seem, therefore, that the increased fall in sensitivity with illumination found in deep red light is not due to increased leakage of current in the cell, as the effect of this would be greater with large external resistance.

(4) A tentative suggestion is put forward that the effect may be due to the occurrence of a threshold for the selenium cell in the neighbourhood of 6600 Å, and that the number of electrons possessing sufficient energies to respond to longer wave-lengths may be so restricted as to increase appreciably the tendency towards saturation noticeable in the current.

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No. 41.

ATMOSPHERIC POLLUTION IN DUBLIN DURING THE YEAR 1940.

By A. G. G. LEONARD, BRIDGET P. McVERRY,
and D. CROWLEY.

[Read April 22. Published separately July 28, 1941.]

Work has been continued during 1940 at Leinster Lawn and the Albert Farm, Glasnevin.

Results obtained with Standard Gauges.

Detailed figures for deposits at Leinster Lawn and Albert Farm are recorded in Tables 1 and 2 respectively.

Soluble solids were deposited at Leinster Lawn at about 74 tons per square mile, and were 1·8 times the quantity at Glasnevin.

Insoluble solids at Leinster Lawn amounted to 113 tons per square mile, and were 4·2 times the quantity at Glasnevin.

The total solids at both stations show a distinct increase over the figures obtained in previous years.

Suspended Impurity and Sulphur Dioxide.

Results of suspended impurity measurements at Merrion Street by the Owens Automatic Filter, and of sulphur dioxide at Leinster Lawn and the Albert Farm by the lead peroxide method, are given in the following table, the sulphur dioxide being recorded as sulphur trioxide:—

	Average Quantity of Suspended Impurity in Milligrams per cubic metre.	Milligrams of SO_3 per 100 sq. cm. of PbO_2 per diem	
		Merrion St.	Albert Farm.
Jan.	0·82	3·39	0 82
Feb.	0·37	1 23	0 76
Mar	0·36	1·10	0 47
Apr.	0 30	—	0·36
May	0·25	0 96	0·26
June	0·16	0·71	0 20
July	0·18	0·79	0·13
Aug.	0·18	0·79	0·13
Sep.	0·21	1·06	0·17
Oct.	0·29	1·74	0 60
Nov.	0·42	2·12	0·44
Dec.	0·47	2·40	0·42

TABLE I.—*Impurity Deposited from the Atmosphere at Leinster Lawn during the Year* 1940.

Grams per Square Decametre (Metric Tons per Hundred Square Kilometres).

—	Rain-fall. mm.	pH of rain-water	Insoluble Matter.			Soluble Matter.		Total Solids.	Included in Soluble Matter			
			Tar.	Carbonaceous other than Tar	Ash	Loss on ignition.	Ash		Sulphates (SO_3).	Chlorine (Cl).	Ammonia (NH_3)	Lime (CaO)
January ..	59	4·4	19	151	252	115	159	696	74	47	3	48
February	90	5·0	23	110	281	110	245	769	82	103	3	55
March ...	31	5·7	12	107	235	34	178	566	50	31	2	44
April ...	74	5·9	12	151	227	102	104	596	81	48	3	51
May ...	20	6·2	7	127	216	24	119	493	36	9	—	26
June ...	24	6·6	8	135	330	102	94	669	40	15	—	42
July ...	102	6·2	9	105	192	82	136	524	59	20	—	45
August ...	12	6·6	8	177	219	62	145	611	42	15	—	37
September ...	47	6·5	7	118	245	63	186	619	47	35	—	57
October ..	134	5·2	11	124	232	97	165	629	80	40	3	53
November ...	86	6·1	7	80	233	79	178	577	62	34	2	41
December ...	44	5·4	20	90	225	140	227	702	97	85	4	54
Mean Monthly ...	60	—	12B	123B	241B	84B	161B	621B	65A	40B	2A	46
Summer Total ...	279	—	51	813	1429	435	784	3512	305	142	3	258
Winter Total ..	441	—	92	662	1458	575	1152	3939	445	340	17	295
Annual Total ...	723	—	143	1475	2887	1010	1936	7451	750	482	20	553

TABLE II.—*Impurity Deposited from the Atmosphere at Glasnevin during the Year 1940.*

—	Rain-fall, mm.	pH of water.	Grams per Square Decametre (Metric Tons per Hundred Square Kilometres).								
			Insoluble Matter			Soluble Matter		Total Solids.	Included in Soluble Matter.		
			Tar	Carbonaceous other than Tar.	Ash.	Loss on ignition.	Ash		SO_4	Cl.	NH_3
January ...	73	4·45	3	24	45	64	103	239	50	32	7
February ...	58	4·54	2	27	40	74	153	296	39	54	2
March ...	38	5·73	2	31	31	38	67	169	24	14	3
April ...	66	5·20	2	28	39	79	61	210	38	18	7
May ...	29	5·60	1	41	41	40	40	163	26	5	—
June ...	17	6·35	1	81	62	76	39	259	12	6	—
July ...	110	6·18	1	54	45	55	72	226	42	9	1
August ...	11	6·44	1	28	61	36	53	182	23	5	—
September ..	49	5·50	1	30	43	41	70	185	31	14	1
October ...	122	4·98	3	39	40	73	116	271	61	28	7
November .	82	5·39	2	39	130	46	63	301	45	21	5
December ...	40	5·78	3	22	28	43	124	220	34	52	5
Mean Monthly ..	58	—	2	37	50	56	82	227	35	21	3
Summer Total ...	282	—	7	262	291	329	335	1225	172	57	9
Winter Total ...	413	—	15	182	314	338	646	1496	253	201	31
Annual Total ..	695	—	22	444	605	667	981	2721	425	258	40

The results obtained in January were the highest so far recorded and were due to the lengthy spell of very cold weather in that month, which gave rise to greatly increased coal consumption.

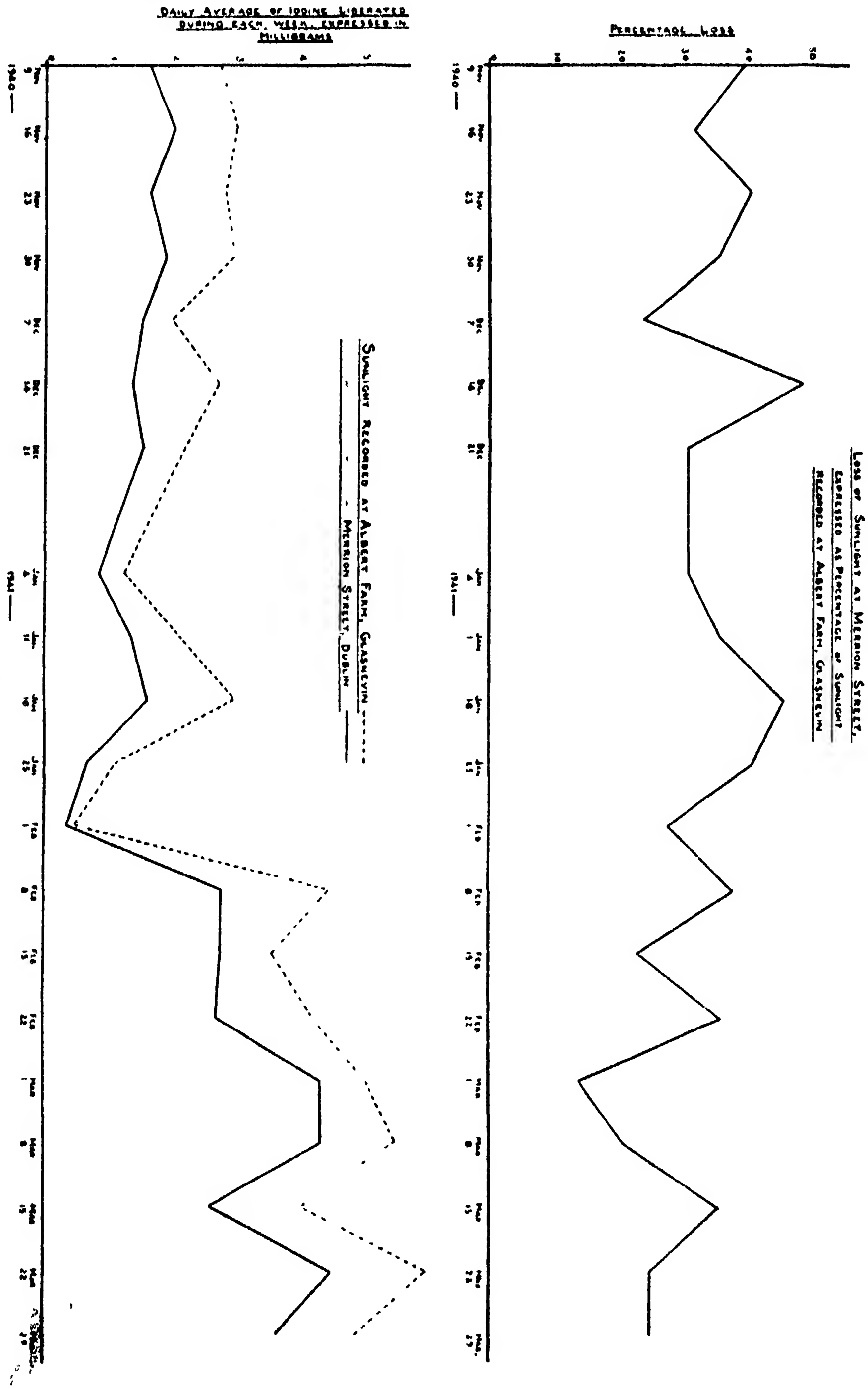

Fig. 1.

Owing to an accident in the laboratory no result was obtained for sulphur dioxide at Merrion Street in April.

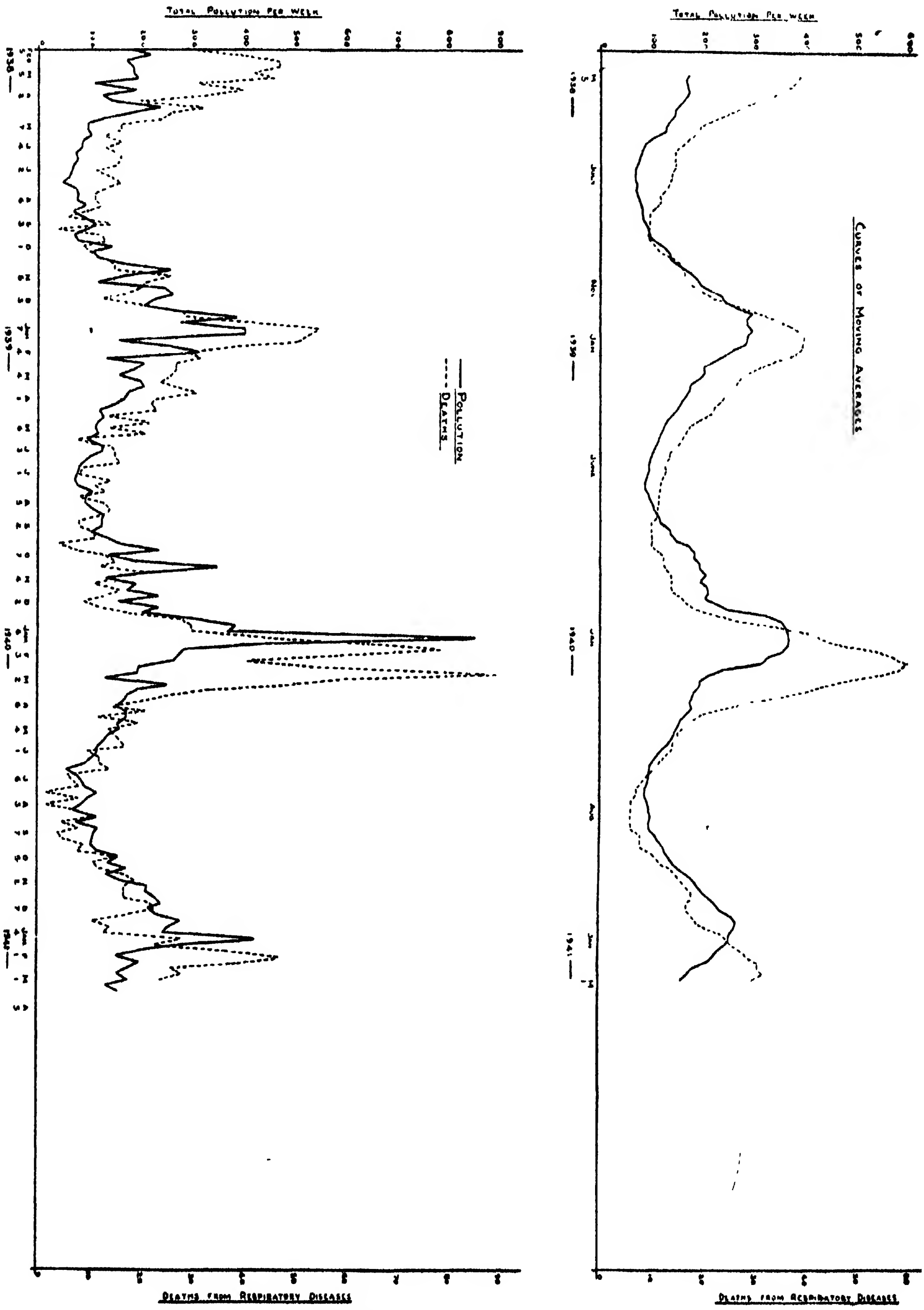

Fig. 2.

Daylight Measurements.

Fig. 1 gives the graphs for daylight measurements by the potassium iodide method at Merrion Street and the Albert Farm. The results are expressed as the daily average of iodine liberated during each week, thereby showing clearly the persistent loss occurring in the city.

The top graph gives the loss of light at Merrion Street plotted as a percentage of the light recorded at the Albert Farm for the same period.

The maximum daily loss of light occurred in the period January 14th to 15th, being 77%.

Pollution by Suspended Matter and Deaths from Respiratory Diseases.

At Dr. Roy Geary's suggestion we have plotted curves of "moving averages" for both these quantities. The points for the graphs are obtained by summing nine consecutive determinations and taking the average; by deducting the first of the determinations, adding the next (the tenth), and dividing by nine a second point is obtained, and so on.

Fig. 2 shows the graphs of moving averages on top with the curves of weekly totals below. The moving average curves smooth out the peaks in the lower graphs very satisfactorily and apparently one should be able to assess the period of lag between the maxima of pollution and deaths. In 1939 the lag was three weeks, in 1940, four weeks, and in 1941 eight weeks, so that it is not feasible to decide on a definite time lag from the data so far available.

The authors wish to express their thanks to Mr. M. G. Dowling for copies of the weekly returns of births and deaths, and to University College for continuation of a grant in aid of the work.

CHEMICAL DEPARTMENT,
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No. 42.

ESTIMATION OF AMMONIA AND UREA BY A MODIFICATION OF THE CONWAY DIFFUSION METHOD.

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[Read April 22. Published separately July 28, 1941.]

In all the experimental work on ureagenesis in the liver, and on kidney efficiency in the healthy and diseased, a large number of urea and ammonia determinations have been the main part of the method of approach. From time to time, therefore, the methods generally used for these determinations have been criticised and modified. The most thorough review and test of this description, which takes account of the diffusion method, is the work by Lee and Widdowson (1). These workers come to the conclusion that from all the current methods tried out, including their own modification, Barrett's method (Nesslerization) is the only one suitable for clinical work. Conway's diffusion method is excluded on the grounds of its specialised technique and apparatus and also for its relatively low range. This opinion has been voiced by other workers as well.

In clinical work the diffusion method of analysis is most desirable, and it is astonishing that so far only one modification of the original method has been suggested, which is also a simplification. As originally described by Abelin (2), this modified method exhibited serious difficulties in the end-point titration. The work submitted here deals with a simple modification, which, in the author's opinion, should make Abelin's method an extremely useful one, especially in clinical research.

Abelin's Method.

In this method the Conway Units are cleansed and prepared in the same manner as described by Conway. The inner compartment is then filled with 1 ml. of 2% boric acid which has just been boiled and is still warm. This boric acid receives one drop of indicator, and the unit is ready for ammonia

absorption. At the end of the period of absorption, the ammonium borate formed is directly titrated with N/100 H_2SO_4 or N/100 HCl from a 2 ml. micro-burette.

There is no difficulty in the preparation of any of these reagents, and the only reagent that must be absolutely quantitatively correct is the N/100 acid; the boric acid need only be approximately 2%, since excess is all that is required. This concentration of boric acid is capable of absorbing a great deal more ammonia than the inner compartment of the unit is capable of holding in equivalent of titration acid. Difficulties will, however, be experienced with the use of Abelin's indicator, which is a Tashiro indicator prepared in the following manner:—Make up a 0 1% alcoholic methyl red and 0·1% alcoholic methylene blue, and mix 100 ml. of the former with 25 ml. of the latter (4 : 1). Take one volume of this solution and dilute it with one volume of alcohol and 2 vol. of CO_2-free water. Add drop by drop N/100 NaOH to this solution until the red colour is just discharged. This preparation is diluted 1 : 9 with CO_2-free water, and one drop of it is added to the boric acid in each unit. Abelin claims that this indicator is green at a pH of 5 6, and red at a pH of 5 3–5 2. 2% boric acid has a pH of 5·2–5 3, and, therefore, is red with the indicator when the unit is set up, changes to green the moment ammonia begins to be absorbed, and is titrated back to red. The results published by Abelin show that duplicate estimations have a negligible titration difference, and that ammonia recovery from standard solutions is for all practical purposes 100%. These results so far have not been confirmed.

Conway, who gave this method a trial in his laboratory, makes the following criticism in his book, "Micro-Diffusion Analysis and Volumetric Error" (3):—"The author has subsequently investigated the method, but considers the end-point unsatisfactory when compared with the barium hydroxide titration." Estimations carried out in this laboratory fully support this view, and to some extent Abelin must have realised the uncertainty of his end-point, because he advises continuing the titration until the colour matches the original colour, which has been preserved in an unchanged blank unit. This, however, is a most unsatisfactory procedure; because once the titration has changed from the green to the faintest red, more acid will only increase proportionally the depth of tint until the colour has arrived at the point where further addition will make no difference to the intensity of red. This makes over-titration easy. The end-point should be decided by colour shade and not by colour intensity.

Abelin's unit when set up contains, roughly speaking (reckoning 20 drops to 1 ml.), 1/80th the amount of Tashiro indicator that the Conway unit contains. The colour produced is so faint that only with prolonged experience can the end-point changes be clearly made out.

These are technical difficulties only, and Conway's comment on the method may rightly be quoted at this point: "The principle is certainly a good one, and if in practice not found particularly good in its present form, may very well prove advantageous by an alteration in the conditions or the reagents."

Alterations in Conditions and Reagents.

All alterations were adopted only after a long period of trial, when they had definitely proved themselves simplifications. No loss of accuracy was incurred by adopting these simplifications, they concern:—(*a*) the indicator, (*b*) the seal, (*c*) the urease preparation.

The Indicator.—It is true that Conway's hydrochloric acid-barium hydroxide titration shows an exceedingly sharp end-point with Tashiro's indicator. Such a sharp end-point can hardly be expected when one is titrating boric acid which contains ammonium borate with N/100 HCl, since the reactions take place in a well-buffered medium, of which the change in pH is a gradual one. The first step taken was to bring the strength of Abelin's indicator up to the strength of Conway's. This indicator demonstrated well the buffered nature of the reaction when one nears the end-point in the titration. The green fades and changes to yellow, the yellow gradually turns orange, and the solution finally is a fairly intense pink. It is obviously difficult to fix an end-point in such a type of colour-change. The next step was to alter the composition of Tashiro's indicator. This was done with a view to increasing the methylene blue screen in order that it might cover the early yellow change of the methyl red. The indicator thus obtained proved to be a double-change indicator: (alkali)-green—blue—cherry red (acid). The indicator was tested in a known pH range of a buffered solution (B.D.H. buffer, according to Prideaux and Ward), and it was shown that the blue phase of the indicator covers the pH range 5·23–5·47. Titrating with this indicator the following colour changes are encountered near the end-point:—The green fades, a drop of acid makes the solution flush deep red, but under stirring (which is absolutely essential) it reverts to green, then becomes almost colourless, and this on closer inspection actually is the blue phase, which then needs either a fraction of a drop or a few drops more to establish an easily recognised permanent pink, which no amount of stirring can get rid of—this is the end-point. These changes are easily recognised if one observes the wall of the inner compartment obliquely instead of vertically. This is done by mounting the unit on a relatively high bench nearly at eye-level of the operator. Thus, delicate colour changes at the periphery of the meniscus can be detected.

The Seal.—Vaseline and a mixture of vaseline and hard paraffin have commonly been employed. In practice both these fixatives make the fingers of the operator greasy. Where incubation is desired, no substitute was found for the vaseline-paraffin mixture (3 : 1). In routine clinical work, however, it should be of advantage not to incubate the units, and in that case the following measures will be found adequate and satisfactory:—Place half the number of the lids used in the experiment on a level table with the ground side up, and put 5–6 drops of liquid paraffin in the centre of each slide, using an ordinary dropping pipette; now, turn the remaining half of the lids on top of the ones

thus prepared A very fine film of paraffin will spread between the ground surfaces of the two lids. The two lids are slid apart when the units are ready to be covered. If the correct amount of paraffin is used the lids will not tend to slide. This method offers three advantages:—Firstly, the operator's fingers remain free of grease during the whole of the experiment; secondly, the fine film of paraffin has rendered the slide perfectly translucent, any change in the reagents can be easily observed; and thirdly, the cleaning of the slides and units is facilitated.

The Urease Extract.—There are two minor practical disadvantages in Conway's preparation of the Urease extract. The glycerol-urease takes a long time to filter (48–72 hrs.) and whenever the filtrate is required, it has to be diluted with phosphate buffer and water. A small variation in the method of preparation produces an extract of equally high qualities, which filters rapidly, and is kept ready for use. Transfer 5 g. of Jack bean meal into an Erlenmeyer flask, add 10 ml. of distilled water, and agitate vigorously for at least 20 minutes, then add 40 ml. of glycerol and 50 ml. of a phosphate buffer of pH 7·4 (3 g. anhyd. Na_2HPO_4 + 2 g. anhyd. KH_2PO_4 in 100 ml H_2O). Shake this mixture well, allow to stand for a minute, and decant the fluid on to a large filter paper. At a temperature of about 20–23° C. filtration will be complete within a short time. Put the filtrate into the ice-box immediately. It can be kept conveniently in a bottle carrying a graduated 1·0 ml. pipette. This extract is ready for use; 0·2 ml. can be run in succession from the pipette into each unit, or a dropping pipette may be used once it has been calibrated. The extract keeps several months.

Suggested Routine Method.

The method here described should be suitable for clinical routine work. The following reagents are required:—

(1) 2% pure boric acid (*Analar*) with incorporated indicator. This is prepared in the following way:—Pipette 0·5 ml. 0·1% alcoholic methyl red and 0·5 ml. 0·1% alcoholic methylene blue into a flask, and add to it 60 ml. of recently distilled water and 20 ml. of alcohol. Now add drop by drop very dilute alkali (N/500 NaOH) until all the red has been discharged and the solution has assumed an olive green colour. 2 g. of boric acid is now added, and the flask is held in a hot-water bath until all the boric acid has dissolved. If the distilled water has not been recently boiled, it is of advantage to bring the solution to a quick boil. (Boiling does not affect the shade or stability of the indicator.) After the flask has cooled, the solution is made up to 100 ml. in a volumetric flask. This solution is of purple colour; the indicator is of the same concentration as that used in the Conway acid.

(2) N/100 HCl.—In making up this acid from an accurate N/10 standard acid it may be of advantage to incorporate some indicator, as a coloured

solution makes the reading of the meniscus in the micro-burette easier, and the concentration of the indicator in the unit is kept the same throughout the titration. To do this proceed as follows:—Measure 1·25 ml. of 0·1% alcoholic methyl red and the same amount of alcoholic methylene blue into a 250 ml. volumetric flask, to this add 50 ml. of alcohol and 150 ml. distilled water, then proceed to bring the solution to the neutral point with dilute alkali, as described for the boric acid under (1). To this neutral solution 25 ml. of the N/10 acid is added, and the flask is filled to the mark with distilled water.

(3) Urease Extract.—The preparation as described.

(4) Saturated Potassium Carbonate.—Dissolve 112 g. in 100 ml. of boiling water, filter while hot. The cold solution should contain some undissolved salt, otherwise add more of the salt.

(5) Liquid Paraffin.—Used as described in the previous section.

The units, which were thoroughly cleaned with running hot water and soap after their last use, are stored in dilute acid, to which some indicator has been added. The first step, therefore, is to take the units required out of the acid and rinse them with running distilled water, they are then inverted on a clean surface such as a filter paper. While the water is thus allowed to run off for some minutes, the lids are prepared with the liquid paraffin as described above. The units are now filled; they need not be completely dry. From a 5·0 ml. graduated pipette run in succession 0·5 ml. of distilled water into each outer compartment. Take a 1·0 ml. pipette graduated to the end of the tip, and run 1·0 ml. of boric acid into each inner compartment; do not blow out the pipette. For one blood urea estimation three units are needed, in two of which is estimated all the ammonia not produced by the hydrolysis of urea, such as the preformed ammonia contained in the distilled water and urease preparation, which is estimated in one blank unit, and the preformed blood ammonia which is estimated in the second blank unit; in the third unit the urea-ammonia is estimated, as well as all the extra-ammonia just mentioned. According to this plan, the outer compartments of the three units receive in addition to the distilled water in order:—"Blank one," 0·2 ml. of the urease preparation; "blank two," 0·2 ml. of the blood; "unit three," 0·2 ml. blood and 0·2 ml. urease preparation. Each unit is immediately closed with the lid. (*Note*: the blood is added before the urease, and the blood pipette *only* is rinsed twice with the distilled water in the outer compartment.) The units are left for at least 20 minutes on the bench to allow for the urease action in unit three; however, no harm results if they are left a little longer. After this time all three units receive 1·0 ml. of the saturated solution of potassium carbonate; this is run into the outer compartment from a 1·0 ml. pipette graduated to the tip. The outer compartment must be uncovered as little as possible for this purpose, and the pipette must not be blown out. The units remain on the bench for 1½ hours at least; titration can be started whenever convenient after this time. The units may even be left on the bench over night and titrated the next morning. The titration is carried out with the N/100

acid from a micro-burette (2 0 ml.). It is absolutely necessary to stir the fluid in the inner compartment with a fine glass rod during titration; the end-point is reached with the first permanent pink in the solution. It is advisable, at least at the beginning, to do all estimations in duplicate. Every ml. of N/100 titration acid corresponds to 0 3 mg. urea or to 0 17 mg. ammonia. The mg. per 100 ml. of original fluid can easily be calculated; for example, using 0·2 ml. blood in the outer compartment—

ml. titration acid $\times$ 0·3 $\times$ 5 $\times$ 100 = mg. urea per 100 ml. of original fluid.

It will be seen from above that the smallest division of the burette (=0·01 ml. acid) corresponds to 3γ urea (=0·003 mg. urea).

"Blank one" (urease ammonia) can be relied upon to be quite negative for the first month at least, after that time a small blank value may be obtained, and the urease solution should not be kept longer than three months. "Blank two" may be omitted altogether for routine clinical work, since blood even after standing for 24 hours has a preformed ammonia value of not more than 1 mg. N/100 ml. This has been pointed out by Conway (Ref. 3, p. 101) and agrees with the present author's experience.

Range and Accuracy of the Method.

The range of the method was investigated first. A 2 "gammil" NH_3–N control solution as recommended by Conway was prepared in order to test the lower limit of the range. This solution is made up as follows:—0 471 g. of analytically pure $(NH_4)_2SO_4$ is dissolved and made up to a litre with distilled water. 2 ml. of this stock is diluted to 100 ml. By making a gradually increasing range of NH_3–N solutions from the stock, the minimum amount of NH_3–N was found which is necessary to change the indicator, or, in other words, the least amount of ammonia which on absorption by the boric acid will shift the pH from 5·23–5 47. This amount was found to correspond to 10γ NH_3–N, and approximately equals the amount of ammonia formed from 0·2 ml. of a solution of 4 mg. % urea. One simple modification would allow one to estimate even more dilute solutions of urea, i.e. the use of 1·0 ml. of analytical fluid in the outer compartment instead of 0 2 ml. This would allow the estimation of 1·0 mg. % urea and slightly less. Incidentally, since 1·0 ml. is the ideal quantity for the outer compartment of the Conway unit, it should be used when obtainable. The upper limit of the range was not sought; 2% boric acid is capable of absorbing a great deal more ammonia than it is possible to titrate with a N/100 acid (HCl) in the inner compartment of the Conway unit. The inner compartment easily will hold 3 0 ml. of titrating acid in addition to the 1·0 ml. of boric acid, always remembering that thorough stirring has to be employed. With some experience, even 4 0 ml. can be safely used. Taking 3·0 ml. as the maximum, the highest level for blood urea (using 0·2 ml.) would be 450 mg. %. This covers the whole range of clinical blood urea values, and no dilution of analytical fluid is needed.

The accuracy of the method was investigated next. For this purpose the routine method of the technique described was employed simultaneously with the original Conway method. 600 mg. of pure urea (Kahlbaum) were dissolved and made up to one litre with distilled water, and a few drops of toluol were added to preserve it. From this stock various dilutions were prepared. Simultaneous duplicate estimations were carried out on these dilutions with both methods. Conway's Range II solutions were employed, i.e. N/200 HCl for the ammonia absorption and N/150 $Ba(OH)_2$ for the titration. The results are given in Table 1. The barium hydroxide titration was done with the standard horizontal burette; in the end-point titration often the fraction of a drop was used. The boric acid was titrated from a 2 0 ml. micro-burette, and no attempt was made to deal with fractions of drops when nearing the end-point of the titration. One drop from the burette when accurately delivered measures 0·01 ml. The reading on the burette was always taken to the nearest 0 01 ml. division. It is therefore not surprising to find the same error throughout the whole range of dilutions, i.e. an error of $\pm 3\gamma$ or at the most $\pm 6\gamma$ which corresponds to $\pm$ 0 01-0 02 ml. of titration acid. It is for this reason that the results, expressed as percentage recovery of added urea, do not clearly reflect the true state of affairs. When doing a large number of estimations over a long period of time, it will be found that by far the most frequent error is $\pm$ 0·01 ml. acid. This will be seen from a representative sample of titrations carried out largely on clinical material or on tissue juices, the figures of which are given in Table 2.

TABLE 1.

γ Urea added	γ Urea recovered by Conway's method.		γ Urea recovered by the Boric acid method.		
	Unit I.	Unit II.	Unit I.	Unit II.	% (aver.).
12	11·60	11·58	15	9	100·00
24	23 00	23 40	21	18	81·25
48	47·20	46·00	48	45	97·00
72	70·80	70·20	72	66	95·83
96	94 60	95 20	96	96	100 00
120	119·20	118·60	117	117	97·50
240			240	243	100·62
360			360	363	100 41
480			480	474	99·35
600			597	600	99·75

TABLE 2.

	ml. N/100 HCl.								
	1	2	3	4	5	6	7	8	9
Unit I. ..	0·36	1·69	0 48	0·48	0·28	0 62	0 31	0·33	0·46
Unit II. ..	0 34	1·72	0 50	0·48	0 27	0·62	0 31	0·34	0·46
Unit III. ..	--	-	—	—	-	—	—	0 33	0·44

It should be possible with the boric method to obtain results as shown in Table 1 quite easily in clinical work. The figures show a standard deviation of 1·5 mg./100 ml. for 0 2 ml. samples, or 0 3 mg./100 ml. for 1·0 ml. samples. Conway in his work (4) shows an accuracy of 0 8 and of 0 52. The error involved as already pointed out is chiefly one of titration, and as the method of titration is refined, the error is decreased. Three steps can be taken in this direction:—one can choose a N/150 HCl instead of N/100, one can deal with fractions of drops near the end-point delivery, and one then can read the meniscus to the nearest half 1/100th division of the 2 0 ml. micro-burette. Duplicate estimations performed under these circumstances are given in Table 3. In order to obtain accurate readings to the 0 001 ml. of titration acid it would of course be simplest to use the N/150 acid in the horizontal burette. The error expressed as standard deviation under these circumstances need not be greater than 0 005 ml. titration acid or 0 5 mg. % if 0 2 ml. of fluid is analysed. Table 3 illustrates this sufficiently. The accuracy of the method compares favourably with Conway's method.

TABLE 3.

	ml N/150 HCl.				
	1	2	3	4	5
Unit I	0 030	0 095	0 170	0·260	0 340
Unit II	0·025	0·100	0·175	0·260	0·340

The advantages of the boric acid method over the original Conway method are many. No special piece of apparatus is required except for the diffusion unit, no alkali-acid factor enters into the calculations, and no dilution of the clinical material is required, since the range covers all possible clinical values. The method should not be employed for urea values below a concentration of 5 mg./100 ml. unless 1 0 ml. of analytical fluid is put into the outer compart-

ment, when one might go as low as 1 mg. %. This drawback certainly is not a serious one for clinical purposes. The direct titration employed in the boric acid method also makes it possible to use two different strengths of acid in the same titration. An increased accuracy is obtained with the N/150 acid when dealing with low values, so it is advisable to start off using this strength of acid. If after having added 2·0 ml. of this acid, the end-point is not reached, one can change over to and continue the titration with the N/100 acid until the end-point is reached. This involves no complicated calculation, since the equivalent amount of urea to every ml. of either acid is exactly known. This is the procedure of choice for research in which urea values between 4 and 400 mg./100 ml. are met with.

SUMMARY.

(1) Abelin's method for the diffusion analysis of ammonia and urea has been investigated.

(2) The method has been found unsuitable in its original form. Certain modifications were tested over a period of 2 years, and a method suitable for clinical routine work as well as for research purposes has been described.

(3) The range of the method lies between 4–400 mg. urea per 100 ml. The recovery of urea by this method has been shown to be quantitative. Under ordinary working conditions with a 2·0 ml. micro-burette the error is ± 1·5 mg. urea/100 ml.; certain refinements will reduce this error to ± 0·5.

(4) The method compares favourably with the original Conway method against which it has been tested.

In conclusion the author wishes to take the opportunity to thank Professors W. R. Fearon and E. J. Conway for the helpful criticism and advice they have given in this work.

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No. 43.

THE PHENOL AMIDINE REACTION: THE DETECTION OF GUANIDINE, GUANIDINE DERIVATIVES, AND UREA, BY MEANS OF THYMOL AND HYPOCHLORITE.

By WILLIAM ROBERT FEARON.

[Read April 22. Published separately July 28, 1941.]

In 1925, Sakaguchi described a reaction characteristic of the monosubstituted guanidines, in which red pigments are obtained when α-naphthol and the guanidine compound are treated with hypochlorite in strongly alkaline solution. Positive results were obtained with arginine, guanidino-acetic acid (glycocyamine), and guanidino-butyric acid; while free guanidine, nitroguanidine, nitroarginine, glycocyamidine, creatine, creatinine, and various urea derivatives yielded no pigments. From this, Sakaguchi concludes (1925, 1) that the test is dependent on the presence of the system $R.NH-C(NH)-NH_2$. Proteins give a positive reaction owing to their arginine content, and the test, suitably modified, has become of value in the estimation of arginine in protein hydrolysis mixtures.

Although Sakaguchi isolated and analysed one of the pigments obtained, he does not appear to have investigated the mechanism and limiting conditions of his test. An early examination made by the present author showed that the α-naphthol could be replaced by other common phenols, provided that they were unsubstituted in the *para* position, and the scope of the reaction has been surveyed by Poller (1927), who reports positive results with dicyandiamide, methylguanidine, and symmetrical dimethylguanidine; and negative results with urea and asymmetrical trimethylguanidine, thus confirming Sakaguchi's conclusions as to the specificity of the reaction.

Recently, in testing for arginine in liver protein extracts, it was observed that the Sakaguchi reaction was untrustworthy, and gave a deep purple colour that could not be matched with the carmine colour given by arginine or arginine-containing proteins, such as caseinogen and gelatin. The purple colour obtained in the liver extract was found to be due to contamination by iron porphyrin, probably haem, which is able to catalyse the oxidation of α-napththol by hypochlorite in strongly alkaline solution. A similar effect with haemoglobin has been noted by Sakaguchi, and made the basis of a delicate test for blood (1925, 2).

With the object of avoiding this extraneous colour production, a reexamination was made of the effect of substituting other and less reactive phenols for the α-naphthol. Out of a number of common phenols examined,

thymol was found to be the most suitable, and by controlling the alkalinity of the medium, the range of the test has been extended so as to include both guanidine and urea, neither of which yields pigments in Sakaguchi's original form of the reaction.

The Thymol Amidine Reaction.

Principle: Compounds containing the amidine group when treated with a minimum amount of hypochlorite in alkaline solution couple with phenols unsubstituted in the *para* position to yield quinonoid pigments. In weakly alkaline solutions (pH 8·5–pH 10), urea, free guanidine, and monosubstituted guanidines yield golden-yellow pigments with thymol. In strongly alkaline solutions (pH > 11), neither urea nor guanidine reacts, and the test is, in general, characteristic of substituted guanidines of the type $R.NH–C(:NH)–NH_2$ or $R.NH–C(:NR)–NH_2$.

Reagents: (1) Thymol in saturated aqueous solution. This contains about 0·3 per cent. of the phenol, and is preferable to stronger solutions in alcohol or other solvents liable to be attacked by the hypochlorite during the course of the test.

(2) Sodium hypochlorite, 2 per cent., representing about 1 per cent. available chlorine.

(3) Sodium carbonate, 2 per cent.

(4) Sodium hydroxide, 20 per cent.

Method: A. *Guanidine Derivatives.*—About 3 ml. of the solution is made strongly alkaline with a few drops of 20 per cent. sodium hydroxide; 3–6 drops of the hypochlorite are added, followed by about 1 ml. of the thymol solution. If the test be positive, a golden-yellow colour develops and persists for several hours.

The test is positive with methylguanidine and other monosubstituted guanidines, including guanidino-acetic acid and arginine, both free and in protein form. It is also given, though more slowly, by asymmetrical dimethylguanidine. The test is negative with symmetrical triphenyl guanidine, creatine, creatinine, dicyanamide, and, under the specified conditions of alkalinity, with guanidine salts and urea, and with all mono- and disubstituted ureas examined.

Although, as might be expected, the thymol reaction is not as rapid or as sensitive as the α-naphthol reaction, it is capable of detecting 0·1 mg. of arginine carbonate in 2 ml. of water. In absence of excess of hypochlorite, the pigments are stable, and, with the exception of those obtained from unhydrolysed proteins, are readily extracted by amyl alcohol.

B. *Guanidine and Urea.*—The guanidine solution, previously neutralised if necessary, is treated with an equal volume of a boric-phosphate buffer of pH 9–pH 10, or with a few drops of 2 per cent. sodium carbonate. A couple of drops of the hypochlorite solution are added. Excess must be avoided, otherwise an orange colour is given by free guanidine in absence of any

phenol. This is the "guanidine-hypochlorite reaction," and will be discussed subsequently. After addition of the hypochlorite and 2 ml. of the thymol solution, on mixing, a yellow colour develops similar to that given by the substituted guanidines, and also extractable by amyl alcohol.

The test for urea is carried out in the same way. Both urea and semicarbazide yield pigments similar to the guanidine pigments, but tending to turn green if the hypochlorite and phenol be in excess, owing to secondary formation of an indophenol pigment.

None of the other monosubstituted and disubstituted ureas examined gave the test.

MECHANISM OF THE REACTIONS.

From an inspection of the formulae of the reacting compounds, the test reveals the presence of the free amidine group $-C(NH)-NH_2$, such as occurs, potentially at least, in guanidine base, urea, semicarbazide, the monosubstituted guanidines, and symmetrical dimethylguanidine. The negative result obtained with creatine offers additional support for questioning the conventional structural formula for creatine, the anomalies of which have been pointed out by Hunter (1928).

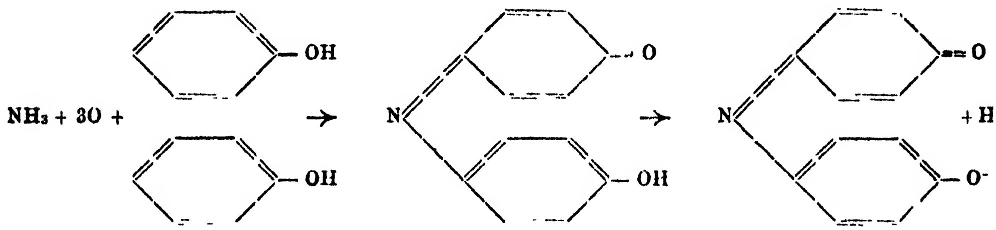
Although the pigments can be separated from the reaction mixtures by means of amyl alcohol, up to the present it has not been possible to obtain them in crystalline form suitable for analysis. Large-scale methods of preparation from the two most readily available sources, namely, guanidine carbonate and urea, have yielded products contaminated with indophenols and thymol oxidation derivatives that are hard to separate. As the indophenol reaction is liable to occur whenever ammonia is generated in presence of a phenol and a hypohalogenite, its conditions and mechanism must be considered.

The Indophenol Reaction.—The reaction appears to have been observed first by Berthelot (1859), who noticed the formation of blue pigments when amino compounds are treated with phenol and hypochlorite. In 1873, Fluckiger described the formation of a similar type of pigment when bromine interacts with phenols in ammoniacal solution. In later years the test has been rediscovered several times (Gibbs, 1926), and it has been adapted successfully for the estimation of ammonia in cerebro-spinal fluid (Thomas, 1912, 1913) and in urine (Orr, 1924). Thymol has been used instead of phenol by Lapin and Hein (1934), and Miller (1935) has employed the reaction as a means of detecting amino acids. In Miller's test, 2 ml. of 0·01 M amino acid solution are treated with 8 drops of 5 per cent. phenol and 2 ml. of 2 per cent. hypochlorite.

A blue colour gradually develops with the common amino acids, the most important exceptions being phenylalanine, tyrosine, tryptophane, histidine, and aspartic acid. Taringi and Lenci (1912) have suggested a mechanism for the general reaction that involves the intermediate formation of a *p*-nitroso phenol,

and its subsequent condensation with another molecule of the phenol to form a phenylimido-quinone.

A simpler explanation appears to be the chlorination of the ammonia, and its subsequent interaction with two molecules of the phenol, one of which is then oxidised to the quinonoid form. The test is given by all ammonium compounds, and by substances, such as urea and guanidine, that liberate ammonia in presence of alkaline hypochlorite. The response obtained with the amino acids in Miller's test can be explained by their relative instability to hypohalogenites, as shown by Werner (1937). The common aliphatic amines do not undergo deamination readily under the conditions of the test, and do not form indophenol pigments.



Indophenol (red)

Indophenol ion (blue)

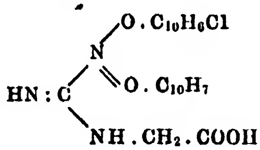
Indophenol formation can be repressed in the phenol amidine test by working with the minimal amounts of hypochlorite and phenol necessary for development of the yellow pigment.

The Phenol Amidine Reaction: (1) *The selective effect of the alkali.*—The degree of alkalinity of the reaction mixture affects both the phenol and the hypochlorite, and, possibly, the configuration of the compound attacked. In solutions more acid than pH 8, both α-naphthol and thymol are oxidised, and may be chlorinated by hypochlorite. α-naphthol yields purple precipitates of complex nature; thymol forms an opalescent precipitate of thymoquinone that turns red on addition of alkali. In weak alkaline solutions of pH 8·5–pH 10, α-naphthol is similarly attacked by hypochlorite, while thymol is much more slowly affected, unless the reagents are in excess. In stronger alkaline solutions of pH > 11, neither the α-naphthol nor the thymol reagent is visibly affected. Hence both reagents may be used for the detection of substituted guanidines in strongly alkaline solution, whereas the α-naphthol cannot be applied satisfactorily for the detection of urea and guanidine, both of which only react in solutions of low alkalinity.

The explanation of this effect of the alkali may be due to the fact that hypochlorous acid is a weak acid, and exists to some extent in the un-ionised form in weakly alkaline solutions, while in solutions more alkaline than pH 11, it is converted into the ionic form ClO^-, which is less effective as an oxidising and chlorinating agent than the un-ionised acid. The amidine group of both urea and guanidine appears to exist in some masked form relatively resistant to the

chlorinating action of ClO^-, but vulnerable to $HClO$, hence the pH of the mixture determines within a fairly narrow range the scope of the reaction. Excess of concentrated alkaline hypochlorite destroys both urea and guanidine (Fenton, 1879), but these conditions do not occur in the amidine test.

(2) *Constitution of the Pigments.*—From 30 gm. of guanidino-acetic acid, Sakaguchi has obtained 2 gm. of the α-naphthol pigment, $C_{23}H_{18}N_3O_4Cl$, which he regards as having the structure:



This formulation, however, does not offer an explanation of the intense colour of the compound, or the fact that it can be bleached by careful reduction with hydrosulphite, and re-oxidised back to the original shade in the manner characteristic of the quinonoids. The colour and general properties of the pigments obtained with thymol, phenol, and other monocyclic reactants closely resemble those of a phenylimido-quinone of the type $O : C_6H_4 : N . C_6H_5$, and it is suggested that the pigments obtained in the reaction are of an imidino-quinone type, in which the two aromatic rings are united to different nitrogen atoms. This suggestion is supported by four observations:

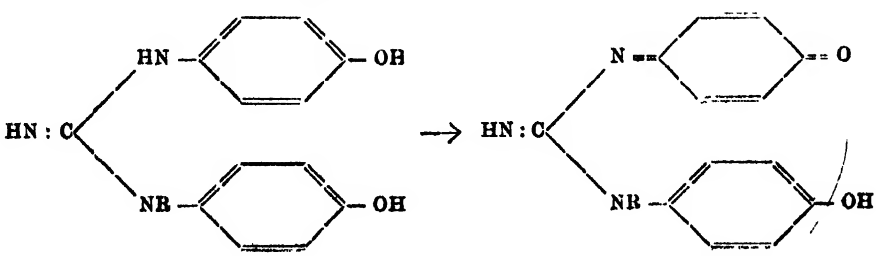
(1) Symmetrical dimethyl guanidine at pH 9–pH 10 reacts with thymol and hypochlorite to yield the characteristic colour. Here, the configuration of the compound does not allow of two phenol groups being coupled to the same nitrogen atom as in Sakaguchi's formula.

(2) Symmetrical diphenyl guanidine at pH > 11, on addition of hypochlorite, in the absence of thymol yields a characteristic yellow colour. If the solution is not sufficiently alkaline, further changes ensue, resulting in a dark brown mixture of pigments.

(3) Substituted phenols in which the *para* position is occupied fail to react, which implies that the coupling in the colour tests involves the *para* position of the reacting phenol.

(4) The test is negative with ammonia, hydrazine, cyanate, aliphatic amines, aliphatic amides, ureides, and all the common amino acids, other than arginine.

Hence it is concluded that the products obtained in the phenol amidine reactions are of the following general type:—



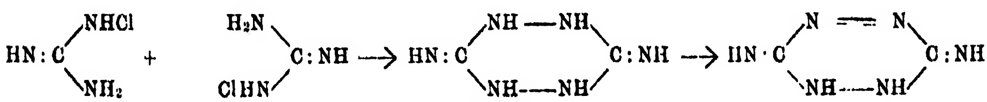
Leuco-form. Pigment.

The yellow colour of the pigments is ascribed to the presence of the imido-quinone system, $O : C_6H_4 : N\text{–}R$, which has been discussed by Stieglitz (1924).

These formulae do not take into account the possibility that one or more chlorine atoms may be present as substituents in the benzene rings, although the fact that the pigments once formed are stable in strongly alkaline solution makes it unlikely that the imino hydrogen has been replaced by the halogen.

The Guanidine Hypochlorite Reaction.—As is well known, guanidine salts under appropriate conditions yield an orange colour with hypochlorite solutions, and this reaction may complicate and confuse the phenol tests.

The hypochlorite reaction, like the thymol reaction, works best in the region pH 8·5–pH 10. If the solution be too alkaline, the colour is not obtained, and for this reason the test is negative when dilute hypochlorite is added to a solution of guanidine carbonate, while the test is positive when hypochlorite is added to a solution of guanidine acetate. The guanidine hypochlorite reaction differs from the thymol reaction in that it requires considerably more than two equivalents of hypochlorite, and the pigment is much less soluble in amyl alcohol. A similar reaction is obtained with semicarbazide and hypobromite, and the pigment has been obtained as an orange, crystalline powder by Lynch (1912), who found it to be a derivative of *p*-urazine. By analogy, the guanidine hypochlorite pigment is either 3 : 6-di-imino-4 : 5-dihydro-1 : 2 : 4 : 5; tetrazine, or a simple chloro derivative.



Pigment.

Neither urea nor any of the substituted guanidines examined yielded a colour with the hypochlorite reagent alone.

Summary.

1. In alkaline solutions of pH 8·5-pH 10, urea, guanidine and mono-substituted guanidines, on treatment with hypochlorite, react with thymol and similar phenols to yield stable yellow pigments of a quinonoid type.

2. In more alkaline solutions of pH > 11, only the substituted guanidines react, and the test becomes a modification of Sakaguchi's α-naphthol reaction.

3. The thymol test is suitable for the detection of arginine, both free and combined in protein form.

4. The conditions and mechanism of the indophenol reaction for ammonia and the hypochlorite test for guanidine are discussed.

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No. 44.

THE HYDROLYSIS OF LAMINARIN.

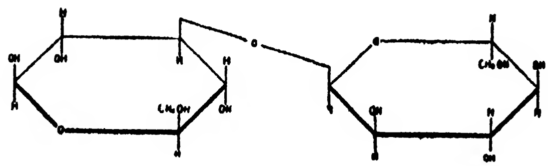
ISOLATION OF A NEW GLUCOSE DISACCHARIDE.

By VINCENT C. BARRY, D.Sc.,

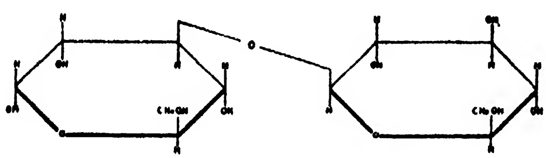
University College, Galway.

[Read May 27. Published separately July 28, 1941.]

Methylated laminarin has been shown to yield on hydrolysis only one trimethyl glucose (1). Unlike the trimethyl glucose resulting from the hydrolysis of methylated starch, cellulose, glycogen, etc., this proved to be 2 : 4 ; 6 trimethyl glucopyranose. The existence in laminarin, therefore, of a linkage quite different from that in the other glucose polysaccharides mentioned above, was evident. It was thus suggested (*loc. cit.*) that it should be possible to obtain from it a disaccharide quite distinct from maltose or cellobiose, and having the structure shown in I or II.



I



II LAMINARIBIOSE

It has now been possible to show that a new disaccharide is present among the cleavage products of laminarin when hydrolysis of the latter is catalysed by enzymes or acids. The disaccharide was first isolated in the form of its phenylosazone, which crystallises in a very characteristic form. Finally, by partial acid hydrolysis of laminarin, the disaccharide was isolated in the form of a syrup,[1] or a white amorphous powder, by fractionation of the products of hydrolysis. It is proposed to name this sugar *laminaribiose*. (Laminariose

[1] On one occasion, after long standing in the air, this syrup crystallised to a considerable extent, forming colourless rectangular prisms, having a melting-point 161–162° C.

was the name given by Kylin (2) to a substance with the formula $(C_6H_{10}O_5)_2 . H_2O$, which, according to him, existed in aqueous extracts of laminariae. No evidence has been found in this laboratory to suggest the existence of any free disaccharide in the aqueous extracts of laminariae.)

As already pointed out (1), the properties of laminarin and its derivatives suggested that it was composed, like cellulose and lichenin, of β-glucose units. This prediction has been confirmed by a study of the behaviour of oligosaccharide fragments of the laminarin molecule towards enzymes. Colin and Ricard (3) showed that laminarin was fermented by snail juice. That observation has been confirmed, but no other enzyme source has yet been found capable of splitting the polysaccharide molecule. Yeast, ptyalin, emulsin, and diastase, all appear to have no action. With snail juice taken from the crop and stomach of *Helix pomatia* or *Helix aspersa* hydrolysis proceeds rapidly at ordinary temperatures, bringing about the conversion of the polysaccharide to glucose. In the course of the hydrolysis it is possible to show the existence in the liquid of laminaribiose by isolating it in the form of the osazone. It is probable, therefore, that the snail juice contains, among other enzymes, a distinct disaccharidase (laminaribiase), which catalyses the breakdown of laminaribiose to glucose. The action of this enzyme must be very rapid, as at no time during the course of the fermentation was it possible to prepare more than a very small quantity of laminaribiosazone from the hydrolysis mixture.

No systematic method of selective adsorption has so far been tried to bring about the elimination of the disaccharidase from the juice. The juice may be precipitated by alcohol and dialysed without altering the nature of its action on laminarin. At temperatures as high as 75° C. deactivation of the disaccharidase is not effected, and glucose is slowly produced from laminarin. Varying the pH from 4·5 to 9·1 altered the rate of hydrolysis, but glucose still remained the main product of the reaction, although when the pH was high on the alkaline side the breakdown was very slow, and the presence of glucose could only be demonstrated after some weeks. Other sources of a laminarin-splitting enzyme were sought. The sea-urchin is supposed to feed on fragments of sea-weed, and it was accordingly thought that an active ferment would be present in this echinoderm. The brown juice obtained from this source was found, however, to be inactive. A similar juice from the periwinkle (*Littorina litorea*)—also a known feeder on the fronds of laminariae—appears to contain no active ferment.[2]

The hydrolysis of the polysaccharide may be easily effected in the cold with concentrated hydrochloric acid or on the boiling water-bath with dilute mineral acid. If the course of the reaction is followed with the polarimeter, it is possible to interrupt the hydrolysis at different stages. With the different

[2] Since this paper was read, a glycerine extract of the limpet (Patella) was found to be capable of hydrolysing both laminarin and starch to glucose.

acids it was possible, at different stages of the reaction, to prepare the disaccharide osazone from the hydrolysis mixture. With a view to separating the laminaribiose N. oxalic acid was found to be the most easily controlled. The reaction was allowed to proceed until it was about two-thirds complete, so as to reduce as far as possible the proportion of oligosaccharides present in the liquid. After neutralisation with chalk the liquid was then fermented by yeast, thus destroying the glucose. The liquid remaining was optically active, having a small positive rotation, and contained laminaribiose mixed with higher

LAMINARIBIOSAZONE.

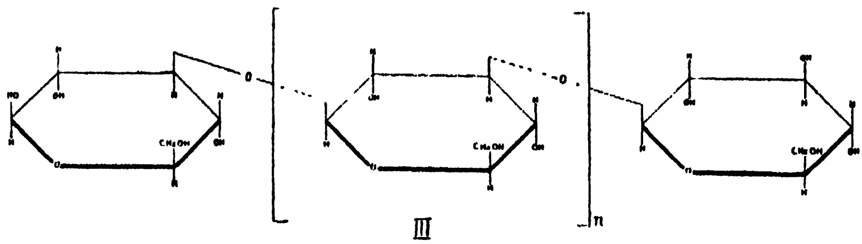
(Magnification = 160 diameters.)

glucose polymers. This mixture, remaining after removal of the water under reduced pressure, was dissolved in methyl alcohol, and fractionated by absolute alcohol and ether. The laminaribiose may be obtained as a white amorphous powder by precipitating with ether from absolute alcohol solution. It has a slightly sweet taste, and yields only glucose on hydrolysis by acid or emulsin. It also forms the beautiful osazone shown in photograph.

If laminarin is two-thirds hydrolysed, as described above, and the oxalic acid then neutralised by chalk, the hydrolysis may now be completed by the addition of emulsin. It is thus clear that the oligosaccharide fragments

resulting from the partial acid hydrolysis of the laminarin are broken down to glucose by the β-glucosidase of emulsin, and so contain β-glucosidic linkages. The laminaribiose itself is rapidly converted to glucose by emulsin, and may accordingly be formulated as glucose-3-β-glucoside (II). If a sufficient quantity of laminaribiose can be accumulated, it will be possible to confirm this structure.

With the isolation of this new glucose disaccharide, and the recognition of the nature of its glucosidic link, it is possible to confirm the structure suggested for laminarin in a previous communication (*loc. cit.*). In III laminarin is formulated as a straight chain of β-glucose molecules linked from the reducing carbon of one hexose to carbon atom 3 of the next hexose molecule.



III

It must, however, be pointed out that from other experiments proceeding in this laboratory it appears improbable that the polysaccharide molecule consists of a simple straight chain.

Zechmeister and Toth (4) found in yeast membrane a glucose polysaccharide with a similar linkage to that occurring in laminarin. They were able, by partial hydrolysis of their substance with strong hydrochloric acid in the cold, to isolate an osazone of a disaccharide. The properties of their osazone are similar to those described here for laminaribiosazone.

Experimental.

Action of Snail Juice on Laminarin.—About 0·5 c.c. of clear brown juice was obtained from the crop and stomach of an edible snail. The alcoholic precipitate from this juice was collected on an immersion glass filter and redissolved in distilled water (2 c.c.). The solution was dialysed for 4 days against distilled water, and then added to a solution of laminarin (1·094 g.) in distilled water (100 c.c.). The rotation of the solution changed from $-0{\cdot}36^\circ$ (initial value) to $+1{\cdot}01^\circ$ (after 3 days). This amounts to 80 per cent. conversion to glucose. The temperature during the fermentation was kept at 12° C. After 24 hours a fraction of the liquid with the usual treatment gave glucosazone in considerable amount. The filtrate after separation of the glucosazone deposited on slow cooling another osazone, which was recrystallised from boiling water. This was the osazone of laminaribiose described below. The yield, however, was small, and was not improved by taking the hydrolysate

at different stages. When juice from the common snail was used the hydrolysis took a similar course. Measurement of the velocity constant for this enzymatic hydrolysis showed that its value falls off as the action proceeds.

When the action of the snail juice was examined at temperatures between 65° C. and 75° C., the hydrolysis was found to proceed more slowly. At 65° the concentration of snail juice required to produce fermentation was about three times that described above. After 24 hours both glucose and laminaribiose were present in the liquid.

When the pH of the hydrolysis mixture was brought to 9 1 by the addition of the calculated quantities of 0·2 M boric acid in 0·2 M potassium chloride and 0·2 M caustic soda, the presence of glucose could not be shown for 21 days. At pH 8·7 the presence of glucose and laminaribiose was demonstrated in the hydrolysate after 6 days. The pH was brought to the acid side using calculated quantities of 0·1 M acid potassium phthalate and 0·2 M caustic soda. Below pH 4, the action of the enzymes appeared to be inhibited. Above this, both glucose and laminaribiose were present in the liquid.

Acid Hydrolysis:

With Concentrated Hydrochloric Acid.—Concentrated hydrochloric acid (250 c.c.) was added to laminarin (30 g.) in a stoppered flask. The laminarin all dissolved in a few hours, and the course of the hydrolysis was followed with the polarimeter. The rotation in a 1 dcm. tube changed from + 1·82° after 20 hours to + 5·68° after 120 hours. The final value taken after 18 days was + 6 65°. When the reading was + 3·95° (48 hrs.), a portion of the liquid was neutralised with barium carbonate, and baker's yeast added to the diluted filtrate. After 48 hours no glucose remained, but the liquid had a small positive rotation (+ 0 26° in a 2 dcm. tube). The liquid was allowed to stand with chalk for some hours, and the yeast cells were then easily removed by filtration. The filtrate was taken to dryness and extracted with hot methyl alcohol. This extract was treated with absolute alcohol until no further precipitate formed after cooling for 24 hours in the refrigerator. The precipitated material was dried in the desiccator to a white powder, which strongly reduced Fehling's solution, but had no sweet taste. It formed an amorphous osazone soluble in hot water, and was probably a mixture of higher glucose polymers. The alcoholic filtrate was taken to dryness and re-extracted with boiling absolute alcohol. Evaporation of the alcohol gave a yellow syrup which had a sweet taste and a low positive rotation, and yielded laminaribiosazone with the usual treatment.

With N Oxalic Acid.—When laminarin is heated with N oxalic acid solution on the boiling water bath, there is no further change in the rotation after 16 hours. Laminarin (2·5 g.) after 7 hours' heating gave 0·5 g. of crude laminaribiosazone after the usual treatment. The hydrolysis was approximately two-thirds complete at this stage. Interruption of the hydrolysis earlier or

later than this seemed to give a smaller yield of disaccharide osazone. Laminarin (32 g.) was heated with N oxalic acid solution (900 c.c.) as described above. After 7 hours the solution (Rotation + 2·87°) was neutralised with chalk, and yeast added to the neutral filtrate. The rotation of the solution had dropped to + 0·17° after 24 hours, and no glucosazone was got with the usual treatment. The liquid was taken to dryness, leaving a light brown syrup. This was redissolved in distilled water (35 c.c.), and 5 c.c. of the solution after decolorising with charcoal were heated on the water-bath with phenylhydrazine acetate. The burner was withdrawn after ½ hour and a yellow osazone separated slowly. This was washed with water, and was then recrystallised from boiling water with slow cooling. The crystals appeared under the microscope shaped like sword-blades, occurring separately and in rosettes. After two further recrystallisations the crystals melted at 195° C. $[\alpha]_D^{10°C.} = -79·6°$ in alcohol. Found N = 10·73 per cent.; calculated 10·77 per cent. The osazone is easily soluble in hot water, alcohol, and in hot acetone. The disaccharide osazone of Zechmeister and Toth (4) had a M.P. 198° C. and $[\alpha]_D^{20°C.} = -75·3°$ in alcohol.

The remainder of the aqueous solution (30 c.c.) above was evaporated to dryness and extracted with boiling methyl alcohol (200 c.c.). This was reduced to half bulk and absolute alcohol added, until no further precipitate was formed (125 c.c.). After 24 hours in the refrigerator, the precipitated material (*a*) was separated by filtration, and the filtrate again taken to dryness. The residue was extracted several times with boiling absolute alcohol, and the combined extracts (125 c.c.) allowed to stand overnight in the refrigerator. A white powdery material (*b*) separated, which was collected and dried. On addition of excess ether to the alcoholic filtrate a white solid appeared, which was filtered on a glass filter, washed with alcohol and ether, and dried in the desiccator to a white powder (*c*). The alcoholic-ether filtrate left a syrup (*d*), when evaporated under reduced pressure.

Properties of these Fractions.

(*a*) This was obtained after drying in the desiccator in the form of a light white tasteless powder easily soluble in water and methyl alcohol. It reduces Fehling's solution, and is easily hydrolysed by emulsin or by warming with dilute acid to glucose. Its aqueous solution does not give any laminaribiosazone after the usual treatment. It has $[\alpha]_D^{15°C.} = +0·3°$ (water), and is thus nearly optically inactive. It is probably composed of laminaritriose mixed with higher polymers.

(*b*) A light white deliquescent powder with a slightly sweet taste. It dissolves easily in cold water and less easily in hot absolute alcohol, from which it separates again on cooling. Its aqueous solution yields some laminaribiosazone accompanied by some less crystalline material. It is easily hydrolysed by dilute acid or by emulsin to glucose. It has $[\alpha]_D^{15°C.} = +15·12°$ (water) 20 minutes

after solution, falling in 24 hours to $+ 13 \cdot 5°$. This fraction weighed about 1 g., and consists of laminaribiose contaminated by oligosaccharide material.

(*c*) This fraction (1 g.) resembles the previous fraction in appearance and properties. Its aqueous solution with the usual treatment gives without recrystallisation beautiful crystals of laminaribiosazone. Hydrolysis with dilute acid or emulsin gives only glucose. This fraction has $[\alpha]_D^{15°C.}$ (water) $= + 20 \cdot 8°$ 25 minutes after solution, falling after 21 hours to $+ 16 \cdot 14°$. The powder softens about 90°, and melts slowly with decomposition to form a milky liquid. The aqueous solution of this fraction on evaporation yields a colourless syrup having a sweet taste. It reduces Fehling's solution, but not Barfoed's solution. This syrup showed no tendency to crystallise.

(*d*) This syrup was small in quantity, and appears to contain some disaccharide. It had $[\alpha]_D^{15°C} = + 5 \cdot 11° \rightarrow 3\ 75°$ after 24 hours in aqueous solution. Hydrolysis yielded a liquid from which glucosazone was prepared in quantity.

The Glucosidic Linkage.—Laminarin (18 g.) was hydrolysed with oxalic acid (400 c.c.), as described above. After 3 hours' heating the solution was neutralised with chalk, and emulsin was added in small quantity to the filtrate. The rotation of the solution gradually increased from $+ 2 \cdot 38$ to $+ 3\ 70°$ in 7 days. No laminaribiose could now be detected in the liquid. In other experiments, laminarin was partially hydrolysed by oxalic acid, and after neutralisation with chalk the glucose was destroyed by yeast. Addition of emulsin to this liquid after removal of yeast caused glucose to be produced in considerable quantity.

The author wishes to express his gratitude to Professor Dillon for the interest he has taken in this work, to Professor S. Shea, M.D., for the photomicrograph of the crystalline osazone, and to the Royal Dublin Society for the loan of a polarimeter. In conclusion, grateful acknowledgment is made to the Industrial Research Council of Ireland, under whose auspices this work was carried out.

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No. 45.

REPORT OF THE IRISH RADIUM COMMITTEE FOR THE YEAR 1940

[Published separately 26TH AUGUST, 1941.]

1,037 tubes containing 8,571 millicuries of radon were issued during 1940, as compared with 1,160 tubes containing 8,630 millicuries in 1939.

The new glass apparatus used in the preparation of these tubes and constructed for the Society in 1939 by Mr. W. Brady in the Chemistry Department of University College, Dublin, has proved very satisfactory in use.

Mr. F. S. Stewart, B.A., was appointed to the Radium Exhibition falling vacant during the year.

Returns from the chief users record the treatment of the following cases with radon or radium element during 1940:—

MALIGNANT CASES.

Dr Oswald Murphy, St. Vincent's Hospital, Dublin ...	91
Dr. Oliver Chance, Richmond, Sir Patrick Dun's, Dr. Steevens', Rotunda, and Coombe Hospitals, Dublin	14
Dr. R. E. Tottenham, City and County Hospital, Londonderry	5
St. Anne's Hospital, Dublin	163
City and County Infirmary, Waterford	19
Galway Central Hospital	8
St. John's Hospital, Limerick	8
Total, ...	308

NON-MALIGNANT CASES.

Dr. Oswald Murphy	80
Dr. Oliver Chance	18
Dr. R. E. Tottenham	3
National Maternity Hospital, Dublin	1
Total, ...	102

The returns show that for the cases of malignant disease first seen during 1940 the numbers treated by the different methods were as follows:—

No. of cases treated	991
" " " by Surgery alone	142
" " " by Radium alone	223
" " " by X-rays alone	505
" " " by Surgery and Radium	20
" " " by Surgery and X-rays	36
" " " by Radium and X-rays	63
" " " by Surgery, Radium, and X-rays ...	2

The following reports have been received, summarising the results of treatments carried out with Radium or Radon (or in some cases X-rays) during the year:—

Report by Oswald J. Murphy, M.B., B.Ch.,

St. Vincent's Hospital, Dublin.

MALIGNANT CASES.

Treated by Surgery and Radium	4
Treated by Radium and X-rays	8
Treated by Radium only	79
	91

Carcinoma of the ear, 1. No glands, 1. Improved, 1.
Carcinoma of the lip, 12. No glands, 9. Improved, 1. Apparently well, 8.
Carcinoma of the cheek, 2. No glands, 2. Improved, 2.
Carcinoma of the parotid, 1. No glands, 1. Improved, 1.
Carcinoma of the tongue, 4. No glands, 0. Anterior third, 1. Well locally. Middle third, 2. Improved, 1. Died, 1. Posterior third, 1. Improved, 1.
Carcinoma of floor of the mouth, 1. No glands, 0. Improved, 1.
Carcinoma of the bladder, 2. Improved, 1. Apparently well, 1.
Carcinoma of uterine body, 1. Apparently well, 1.
Carcinoma of uterine cervix, 7. Group I, 5. Apparently well, 4. Group II, 2. Apparently well, 1. Improved, 1.
Carcinoma of the skin surfaces, 19. No glands, 19. Improved, 6. Well, 13.
Rodent ulcers, 41. Well, 28. Improving, 12.

NON-MALIGNANT CASES.

Hodgkin's disease, 2. T.B. cervical glands, 6. T.B. peritonitis, 8. Uterine hæmorrhage, 2. Angiomata, 20. Lupus vulgaris, 3. Lupus Erythematosus, 1. Keloids, 4. Dupeytron contraction, 1. Pruritis vulvae, 1. Warts, 32. Total, 80.

Report by Oliver Chance, M.B., B.Ch., D.M.R.E.,

Richmond, Sir P. Dun's, Dr. Steevens', Rotunda, and Coombe Hospitals.

During 1940 most of the cases requiring radiation seen in the above hospitals were referred to St. Anne's Hospital for treatment. The bulk of the cases included below were either private patients or else suffering from such conditions as Rodent Ulcers for which they could be treated as out-patients.

Altogether 132 malignant patients and 41 patients suffering from benign conditions were treated by radiation.

The following is a list of the cases in which radon was used:—

MALIGNANT.	
Carcinoma cervix uteri	2
Carcinoma body of uterus	1
BENIGN.	
Toxic goitre	9
T.B. peritonitis	6
Menopausal hæmorrhage	2
Naevus	1

The increasing use of Contact X-rays in cases of superficial malignant disease is largely responsible for the great decrease in the amount of radon used.

Report by R. E. Tottenham, M.D., B.Ch., B.A.O.,

City and County Hospital, Londonderry.

I only see the cases of uterine cancer, and those occurring in other parts of the body do not come under my care. The following five cases were treated:—

Mrs. McK.—Carcinoma of the cervix.

Three applications as follows:—1st, 2,394 M.E.H. 2nd, 1,729 M.E.H. 3rd, 2,793 M.E.H. Total, 6,916 M.E.H.

For about six months her condition was satisfactory. She then developed an abscess in the lower abdomen, which had to be opened. Her health at the

moment (July, 1941) is very poor (i.e. 1½ years after treatment). She also appears to have an old standing tubercular lesion.

Mrs. C. D.—Carcinomatous ulcers of vulva.

This patient received in all about 2,000 M.E.H. in interrupted periods, the radon being applied in needles. The results were not very satisfactory.

Mrs. M.—Columnar celled carcinoma of cervix.

Initial course of approximately 8,000 M.E.H. Condition showed improvement for a short time. Five months later there was a recurrence.

A second course of smaller dosage was given, which was followed by improvement for about three months, when more recurrences appeared. The patient has since died.

In my experience columnar celled carcinomas are not very radio sensitive.

I have lost trace of the other two cases who have gone to the country; they were both columnar celled type.

I have used radon with some success in the treatment of non-malignant hæmorrhages of middle age, the radon capillaries being inserted into a round brass container about an inch and a half long. This container has thick walls, and is about the diameter of the cap of a fountain pen. It is pushed up into the uterus.

Report from St. Anne's Hospital, Dublin.

Radiologist: Dr. Oliver Chance.

163 cases were treated with radon or radium element. Of 41 cases of carcinoma of cervix uteri seen at above hospital a total of 36 were treated, viz., 12 with radium or radon alone, 4 with X-rays alone, and 20 with radium and X-rays combined. 17 are known to be alive, 4 are known to be dead, and 15 cases are untraced.

Report from St. John's Hospital, Limerick.

Radiologist: H. G. Roche Kelly, M.D., D.P.H.

Number of cases treated during the year 1940, 8. All these were cases of rodent ulcer of the face situated on the cheek, temple, forehead, or eyelid. Treatment was by insertion of seeds in margin (4 cases) or by surface application in applicators built up from adhesive plaster (4 cases). In all cases complete healing resulted.

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PHOSPHATE SEPARATION IN QUALITATIVE ANALYSIS.

BY PROFESSOR J. REILLY AND M. O'BRIEN, M.Sc.,
Chemical Department, University College, Cork.

Price One Shilling.

No. 47.

PHOSPHATE SEPARATION IN QUALITATIVE ANALYSIS.

By PROFESSOR J. REILLY and M. O'BRIEN, M.Sc.,
Chemical Department, University College, Cork.

[Read November 25, 1941. Published separately February 11, 1942.]

In the schemes of qualitative analysis most widely used it is necessary to remove phosphate ions, if present, before the iron-aluminium group of metals can be separated from the alkaline earth group. Of the various methods proposed for the removal of the phosphate ion, only two are widely used, namely the tin method and the ferric chloride method. These are to some extent unsatisfactory. They all have certain disadvantages—such as incompleteness of removal, difficulty of technique, loss of other ions, etc. For example, the tin method gives incomplete removal, except when a large amount of tin is used. On the other hand, the use of large amounts of tin causes an appreciable loss of other ions. Curtman (1) found that if more than 2 g. of tin were used there was almost complete loss of iron and barium. The ferric chloride method is generally recognised as a long operation, involving large volumes and giving incomplete removal unless carried out with great accuracy. In this method also there is loss of other ions (1).

The zirconium oxychloride method, which was devised by Curtman (1), is more satisfactory than either of the above. Removal by this method depends on the fact that zirconium forms a phosphate which is insoluble in strongly acid solution, and hence the phosphate ion can be removed from solution by the addition of a soluble zirconium salt. The reagent used in removal by this method is zirconium oxychloride ($ZrOCl_2$).

To the filtrate from the copper and tin groups add 2 g. of ammonium chloride and 10 ml. of zirconyl chloride solution (50 mg. of Zr . . . . per ml.) drop by drop, stirring vigorously. Add 0·2 g. of asbestos or one Fisher filtration accelerator. Render alkaline with ammonium hydroxide, and boil for 2 minutes. Neutralize with dilute hydrochloric acid, add 10 ml. of 3N hydrochloric acid in excess, and boil for 3 minutes. Filter hot on a fluted filter.

Addition of the zirconium oxychloride solution gives a white insoluble precipitate of zirconyl phosphate. The reagent is added dropwise, as rapid addition causes incomplete removal, and the precipitate formed is usually colloidal (2). The solution is agitated during addition to prevent colloidal formation. Excess zirconium is co-precipitated with the phosphate as zirconium hydroxide by adding ammonium hydroxide and boiling. The metals of the iron-aluminium group are also precipitated at this stage. Curtman, however, claims (3) that on boiling with hydrochloric acid in the next step, the

zirconium hydroxide does not redissolve, and in this way excess zirconium can be removed. Pittmann refutes this claim (2), and asserts that it will completely dissolve under the conditions of the experiment.

The following (2) are the main disadvantages of the Curtman procedure:—

1. Notwithstanding dropwise addition of the reagent, agitation of the solution, and the presence of ammonium chloride, the precipitate is largely colloidal. This introduces difficulty in filtering.
2. There is a large loss of other ions.
3. Usually there is a large excess of reagent, which interferes in later tests.
4. The method of removal of excess reagent is unsatisfactory. Most of it comes down with the iron-aluminium group, and interferes in the detection of these metals.

In an effort to eliminate these difficulties, Pittmann proposed the following modified procedures:—

The filtrate from the acid hydrogen sulphide precipitation (copper-tin group) is boiled until all hydrogen sulphide has been expelled, and the volume of the resulting solution is adjusted to approximately 100 c.c. The cooled solution is neutralized with ammonium hydroxide, and then made acid with 5 c.c. of 6N nitric acid. To this acid solution 35 c.c. of 0·015 M zirconyl chloride solution (for every 40 mg. of phosphate) are added, a few drops at a time, with vigorous stirring during the entire addition. The stirring is continued for a few seconds, and then the precipitate is allowed to settle for 1 minute. The mixture is filtered on a very porous filter paper, such as Delta 366, without suction. As much of the supernatant liquid as possible is poured through the paper before the main body of the precipitate is added. The precipitate is washed several times with small amounts of cold water, or, even better, with a 5 per cent. solution of ammonium nitrate, and the washings are collected in the same beaker with the main portion of the filtrate. The precipitate is rejected. The solution now contains less than 1 mg. of phosphate, and in most cases the small amount remaining can be disregarded. However, if it is desired to remove the last traces, 10 c.c. of 0·05 M zirconyl chloride solution are added to the filtrate, and the mixture is heated to just below boiling and held at that temperature for about 2 minutes. The solution is allowed to stand for 5 minutes and filtered.

The aim of the present work is to investigate the efficiency of the Pittmann procedure. It will be shown that it has disadvantages. In an effort to overcome these the factors responsible for them have been studied and eliminated, and a modified procedure for the removal of phosphate by means of zirconium oxychloride has been proposed.

Experimental.

Several phosphate removals were made by the Pittmann procedure, and in each case the following points were noted:—nature of precipitate, time of filtration, nature of filtrate, amount of excess reagent, volumes involved. The percentage removal was also determined by estimating the phosphate in the filtrate after removal. This was done by the hydroquinone colormetric method (4), using a Klett colorimeter. The efficiency of this method of phosphate estimation was first tested with standard phosphate solutions.

The result of many experiments showed that the Pittmann procedure has the following disadvantages:—

1. There is incomplete removal, only 94 per cent. (approx.) of the phosphate being removed.

2. Notwithstanding careful addition of the reagent and vigorous stirring throughout, the phosphate precipitate is, as in the Curtman procedure, largely colloidal. Pittmann asserts that it settles quickly, and filters easily. The contrary has been found to be the case in the present investigation. In no case did the precipitate completely settle, even after allowing it to stand for 2 hours.

3. On account of the weak solution of zirconyl chloride employed (0·48 per cent.), and due to the fact that the filtrate from group 2 must be adjusted to 100 c.c. before removal, large volumes are involved, which necessitate tedious evaporation after removal.

4. Due to colloidal formation, and the large volumes involved, much difficulty is encountered in filtering. Moreover, the filtrate is cloudy.

5. Precipitation of excess zirconium with ammonium hydroxide showed that considerable excess of reagent is used. Apart from the consideration of cost, the use of excess zirconyl chloride is a disadvantage, as it interferes in the detection of the iron-aluminium group.

6. The Pittmann procedure presumes that the amount of interfering phosphate present is approximately known, which in most analyses is not the case.

For a satisfactory method of removal of phosphate by means of zirconium oxychloride, the following conditions must be fulfilled:—(*a*) complete removal, (*b*) elimination of colloidal precipitation, (*c*) small volumes, (*d*) absence of large excess of reagent, (*e*) no serious loss of other ions. The following investigation was undertaken in order to obtain these conditions.

It has already been stated that large volumes are involved in removal by the Pittmann procedure. The following example will illustrate this:—

In carrying out his instruction as to the volume of reagent to be added (35 c.c. for every 40 mg. of phosphate), a solution containing 0·14 g. of phosphate required 122·5 c.c. of the reagent. The volume of the filtrate after removal of the phosphate was approximately 240 c.c.

The use of large volumes is undesirable for the following reasons:—

1. As the reagent must be added dropwise, and with vigorous stirring, addition of large volumes is a slow and tedious operation. It was found, as a result of several trial runs, that addition of the reagent in the removal of 0·14 g. of phosphate takes *at least 30 minutes.*

2. Owing to the fact that the phosphate precipitate is largely colloidal, and does not settle, the use of large volumes increases the difficulty of filtration.

Filtration after removal of 0·14 g. of phosphate took approximately 1½ hours in every case.

3. The use of large volumes necessitates tedious evaporation after removal. For example, after removal of 0·14 g. of phosphate, the filtrate must be reduced from approximately 240 c.c. to 10 c.c. (approx.) in order to obtain a volume which can be conveniently handled in the analysis for the remaining metals.

(A) In an effort to accelerate the removal and obviate evaporation, the following procedure was adopted:—

(*a*) Instead of adjusting the volume of the filtrate from Group 2 to 100 c.c. a volume of approximately 20 c.c. was used.

(*b*) A 10 per cent. solution of zirconium oxychloride was used instead of a 0·48 per cent. solution (0·015 M), as used in the Pittmann procedure. 10 c.c. of the reagent was added.

The following results show that the modified procedure was a distinct improvement:—

		Time of addn. of reagent.	Time of filtration.	Vol. of Fil. after removal.	Time of removal.
Pittman Procedure	...	30 mins.	85 mins.	240 c.c.	2 hrs.
Modified Procedure	...	2½ mins.	20 mins.	40 c.c.	27½ mins.

(B) A serious disadvantage of the Pittmann procedure is the fact that the phosphate precipitate is largely colloidal. This makes filtration very difficult, and gives a cloudy filtrate.

In order to ascertain the stage at which colloidal precipitation began, 0·14 g. of phosphate (as potassium dihydrogen phosphate) was removed by the Pittmann procedure, but the precipitate was allowed to stand for a few minutes after addition of every 10 c.c. of the 0·015 M zirconyl chloride solution, and examined. It was observed that after addition of 80 c.c. of the reagent, the precipitate was granular, and settled quickly. On adding a further 10 c.c., however, colloidal formation was distinctly indicated. The mixture now became milky; the precipitate lost its granular appearance, and did not settle after allowing it to stand for 30 minutes.

The same weight of phosphate (0·14 g.) was again precipitated, but this time only 80 c.c. of the reagent was added. The mixture was now filtered, time of filtration noted, nature of filtrate observed, and the filtrate was boiled with ammonium hydroxide, to precipitate excess zirconium, if present. The results

are given in the following table, together with the corresponding results after addition of the volume of reagent prescribed by Pittmann (122 5 c.c.).—

		80 c.c. of 0·015 M $ZrOCl_2$ solution added.	122·5 c.c. of 0·015 M $ZrOCl_2$ solution added (Pittmann procedure).
Nature of precip.	...	granular	largely colloidal
Time of filtration	...	25 minutes	85 minutes
Nature of filtrate	...	clear	cloudy
Excess reagent	...	very slight excess	large excess.

The fact that the filtrate after removal of the phosphate by addition of 80 c.c. of the reagent gave a very slight precipitate of zirconium hydroxide on boiling with ammonium hydroxide, showed that 80 c.c. of the 0 015 M zirconyl chloride solution was just sufficient to completely precipitate the phosphate. It also showed that a considerable excess of reagent is used in the Pittmann procedure, and suggested that *colloidal formation might possibly be due to this excess.*

(C) The stage at which colloidal formation begins during removal by the modified procedure (i.e. adjusting the volume of the filtrate from Group 2 to 20 c.c., and adding a 10 per cent. solution of zirconyl chloride) was now investigated. 0·14 g. of phosphate was again used, and the 10 per cent. solution of the reagent was added dropwise and with vigorous stirring. After adding 3·5 c.c. of the reagent, the mixture was filtered. A further 0·2 c.c. was now added to the filtrate, and the mixture was again filtered. This filtrate was again treated with 0·2 c.c. of the reagent, and filtered, and the operation was repeated until precipitation was complete. In each case the nature of precipitate and filtrate was observed.

The following results were obtained:—

(*a*) Phosphate soln. + 3·5 c.c. reagent—granular ppt., clear filtrate.
(*b*) Filtrate from (*a*) + 0 2 c.c. reagent— " " " "
(*c*) " " (*b*) + 0·2 c.c. " — " " " "
(*d*) " " (*c*) + 0·2 c.c. " —no pption. or cloudiness.

Addition of a further 3 c.c. of reagent to the filtrate from (*c*) gave no precipitation or cloudiness.

3·7–3·9 c.c. of a 10 per cent. solution of zirconyl chloride *just* precipitates 0·14 g. of phosphate. Note that no colloidal precipitation was observed in this experiment. Note also that, although a considerable excess of reagent was added, at no time was there excess of reagent with the phosphate precipitate. This strengthens the possibility that colloidal formation is due to excess reagent.

(D) 0·14 g. of phosphate (in a solution of 20 c.c.) was now *just* precipitated by addition of 3·9 c.c. of the 10 per cent. zirconyl chloride solution, and the

mixture was filtered after allowing it to stand for a few minutes. This resulted in

(*a*) A heavy granular precipitate which settled quickly, giving a clear supernatant liquid.
(*b*) Time of filtration—7 minutes.
(*c*) Clear filtrate.

In order to see the effect of excess reagent on the phosphate precipitate, the same weight of phosphate (0·14 g.) was again *just* precipitated by addition of 3·9 c.c. of the 10 per cent. solution of zirconyl chloride. A heavy precipitate was again obtained, which settled quickly, to give a clear supernatant liquid. 3 c.c. (excess) of reagent was now added dropwise and with vigorous stirring. Distinct colloidal formation was observed; the precipitate lost its granular nature, and a milky "solution" was obtained. The precipitate did not now completely settle, even after allowing it to stand for several hours. Filtration was very difficult, and a cloudy filtrate was obtained.

The results of Experiments **B, C,** and **D** prove conclusively that *colloidal precipitation is due to excess reagent.*

Pittmann states (2) that if precipitation is made from a hot solution, or if precipitation is made from a cold solution, and the mixture then heated to boiling, the tendency is very markedly towards the formation of a colloidal suspension. This was tested for the case where large excess of the reagent is not added.

(E) 0·14 g. of phosphate was just precipitated from a hot solution by addition of 3·9 c.c. of a 10 per cent. solution of zirconyl chloride. As a result—

(1) No colloidal formation was detected. A heavy precipitate was formed, which settled in a few minutes, to give a clear supernatant liquid.

(2) The mixture filtered in 5 minutes, and a clear filtrate was obtained.

(F) 0·14 g. of phosphate was then just precipitated from a *cold* solution by addition of 3 9 c.c. of the 10 per cent. solution of zirconyl chloride. The mixture was heated to boiling, and then allowed to stand for a few minutes before filtering.

(1) No colloidal formation was detected. The phosphate precipitate settled quickly, and the supernatant liquid was clear.

(2) The mixture filtered in 7 minutes, giving a clear filtrate.

(G) After removal of 0·14 g. of phosphate (in 20 c.c. of solution, as in **C, D, E,** and **F**) by addition of 3·9 c.c. of the 10 per cent. solution of reagent, the mixture was boiled for half a minute, and filtered hot. The time of filtration was 2½ minutes. Clear filtrate.

The results of experiments **E** and **F** show that, contrary to Pittmann's assertion, neither precipitation from a hot solution, nor heating of the mixture to boiling after precipitation in the cold, causes colloidal formation, *provided*

excess of reagent is not added. It is seen from experiment **G** that boiling the mixture after precipitation and filtering hot considerably accelerates filtration.

Pittmann states that if the solution is not agitated during precipitation, the tendency is toward formation of a colloid (2). This was now tested for the case where excess of reagent is not added.

(H) 0·14 g. of phosphate was just precipitated by addition of 3·9 c.c. of the 10 per cent. zirconyl chloride solution. The reagent was added dropwise, but *without stirring.* A heavy precipitate was obtained, which settled *immediately.* On boiling the mixture and filtering hot a clear filtrate was obtained. Time of filtration—3 minutes.

In the foregoing investigation, many factors which were supposed to influence colloidal formation have been studied. It has been proved to be due solely to addition of excess reagent. In previous investigations this cause seems to have been overlooked. It is seen that, provided excess of zirconyl chloride is not added, neither precipitation from a hot solution, nor boiling after precipitation in the cold, will cause difficulty due to colloidal formation. On the contrary, boiling the mixture after precipitation, and filtering hot, enables the filtration to be carried out in a few minutes (Expt. **G**). It is also seen that agitation of the solution is unnecessary when excess of reagent is not added (Expt. **H**).

Is the phosphate completely removed by the Pittmann procedure? This important question was next studied. The percentage removal was determined as follows:

(K) A known weight of phosphate (0·14 g.) was dissolved in water, and, after removal of the phosphate in strict accordance with Pittmann's instructions, the bulk of the filtrate was reduced on a water-bath, and the volume then accurately adjusted to 100 c.c. The amount of phosphate in the filtrate was then estimated by the hydroquinone colorimetric method (4), using a Klett colorimeter. The efficiency of this method of phosphate estimation was first tested with standard phosphate solutions, and it was found to be sufficiently accurate for the purpose.

The results showed that approximately *6 per cent. of the phosphate is unremoved.* The immediate problem, therefore, was to discover the cause of incompleteness of removal.

(L) In the latter experiment the phosphate was removed by addition of 122·5 c.c. of an 0·015 M zirconyl chloride solution in accordance with Pittmann's instruction (35 c.c. for every 40 mg. of phosphate). Removals of the same weight of phosphate (0 14 g.) were now carried out by the addition of (*a*) 80 c.c., (*b*) 100 c.c., (*c*) 155 c.c., (*d*) 175 c.c., (*e*) 200 c.c., respectively, of the 0·015 M zirconyl chloride solution, and the percentage phosphate unremoved was determined in each case by the method already described. The following important results were obtained:—

Volume of 0·015 M $ZrOCl_2$ Solution added.	Percentage phosphate unremoved.
80 c.c.	0·2
100 c.c.	2·4
122·5 c.c.	5·9
155 c.c.	7·1
175 c.c.	7·4
200 c.c.	7·7

It is seen that (*a*) 80 c.c. of the reagent causes practically complete removal, (*b*) the percentage phosphate unremoved increases with the volume of reagent added in excess of 80 c.c.

It has already been found (Expt. **B**) that 80 c.c. of an 0·015 M zirconyl chloride solution just precipitates 0·14 g. of phosphate. It is clear, therefore, that incomplete removal is caused by addition of excess reagent. The fact that the percentage of phosphate unremoved increases with the amount of excess reagent added suggests that this excess of zirconyl chloride forms some soluble complex with the phosphate precipitate. On plotting a graph showing the relation between the volume of reagent added, and the percentage phosphate unremoved, a smooth curve was obtained. A study of the curve showed that the percentage phosphate unremoved approaches a limit (roughly 8 per cent.). This would be explained by the assumption that excess zirconium oxychloride forms a partly soluble complex with the phosphate precipitate. The point at which the maximum amount of phosphate is in solution is reached when the amount of excess zirconyl chloride added is sufficient to convert *all* the insoluble phosphate precipitate into the partly soluble complex. It follows that any further addition of reagent will have no effect on the amount of phosphate in solution.

(M) The completeness of removal by the modified procedure was now examined. 0·14 g. of phosphate (as potassium dihydrogen phosphate) was dissolved in water, and the volume of the solution adjusted to approximately 20 c.c. The phosphate was now just precipitated, by slow dropwise addition of 3·9 c.c. of a 10 per cent. solution of zirconyl chloride, with vigorous stirring throughout the operation. The mixture was then heated to boiling, and filtered hot. The precipitate was washed thoroughly, and the washings collected with the main filtrate. The volume of the filtrate was now accurately adjusted to 100 c.c., and the amount of phosphate present estimated by the hydroquinone colorimetric method. No colour could be detected in the colorimeter. As the method is claimed to be sufficiently sensitive for the estimation of 0·01 mg. of phosphate, the result showed that more than 99·9 per cent. of the phosphate had been removed.

Conclusion. Excess reagent causes incomplete removal. The phosphate is completely eliminated only when excess reagent is not added.

It has been shown that two of the most serious disadvantages of the zirconium oxychloride method of phosphate removal, as carried out by Curtman and Pittmann, namely, incomplete removal and colloidal formation, are due to excess reagent. As a result of the foregoing investigation, the following conditions have been found to give complete removal of the phosphate in an easily filterable condition, and to obviate tedious evaporation after removal:—

(1) Adjust the volume of the filtrate from Group 2 to approximately 20 c.c.

(2) Add a 10 per cent. solution of zirconyl chloride, a few drops at a time, until the phosphate is *just* completely precipitated.

As the success of the method, from the standpoint of efficiency and ease of execution, depends on the condition that excess of reagent is not added, the problem now was to devise a means by which the point of complete precipitation could be ascertained.

(N) The various adsorption indicators—fluorescein, eosin, tartrazine, etc.—were used without success. None of them gave a colour change on complete removal of the phosphate.

(P) The possibility of being able to detect the point at which addition of one drop of the reagent gave no precipitate, by means of a coloured background, was then studied. The filtrate from Group 2 was highly coloured by different colouring agents (e.g., methylene blue, magenta, etc.), and the reagent was added drop by drop. It was found that when the solution was coloured deep blue or brown, the point of complete precipitation could be satisfactorily determined. Over the dark-coloured background of the solution, the white colour of the precipitate formed on addition of each drop of reagent was plainly visible momentarily, but was destroyed on stirring. The procedure, therefore, was to produce a deep blue or brown coloration of the solution by addition of some suitable colouring matter. Then add the reagent, a few drops at a time, stirring after each addition, until addition of a few drops causes no temporary white coloration on the surface of the solution. This method was, however, deemed unsatisfactory, as it necessitates the introduction of foreign matter into the solution, which is undesirable. Moreover, the heavy colour of the solution must be destroyed, as it interferes in later stages of the analysis.

(Q) The following procedure for determining the end point was then tried, and found to be very satisfactory:—

The filtrate from Group 2 was transferred to a boiling tube (small type), and the volume adjusted so that it filled approximately half the tube. The 10 per cent. zirconyl chloride solution was then added from a burette, a few drops at a time, *without stirring.* The precipitate formed on addition of each few drops settled immediately. After each addition the precipitate formed was allowed to settle before further addition. The end-point was reached

when addition of a few drops of the reagent caused no precipitation. The aim in using the boiling tube was to get a large depth of solution with a volume of 20 c.c.–30 c.c., so that an appreciable column of clear supernatant liquid would be obtained during precipitation. One difficulty encountered was that the precipitate, being very flocculent, sometimes completely fills the solution before the end-point is reached, giving no supernatant solution. This was overcome by the following procedure:—If the precipitate becomes too bulky before the end-point has been reached, or if it does not completely settle, boil the mixture for one minute and allow to stand. The precipitate will now settle in a few minutes in much smaller bulk, giving an appreciable column of clear supernatant solution.

As 4 or 5 drops (approx. 0·5 c.c.) of excess reagent might possibly be added by this method, the extent to which this small excess affects (*a*) completeness of removal, (*b*) colloidal formation, was examined.

(R) After adjusting the volume of a solution containing 0 14 g. of phosphate to half-fill a small boiling tube, *4 6 c.c.* of a 10 per cent. solution of zirconyl chloride (approx. *0·7 c.c. in excess*) was added dropwise, without stirring. The mixture was then heated to boiling, and filtered hot. The amount of phosphate in the filtrate was estimated by the hydroquinone colorometric method.

(*a*) Time of filtration—5 minutes.
(*b*) Clear filtrate.
(*c*) 99·6 per cent. of the phosphate was removed.

(RI) The same weight of phosphate was removed by the same method, but this time 5 c.c. of the reagent (approx. 1·1 c.c. in excess) was added.

(*a*) Time of filtration—5 minutes.
(*b*) Clear filtrate.
(*c*) 99·3 per cent. of the phosphate was removed.

The results of experiments **R** and **RI** show that *small excess of reagent does not affect the efficiency of this method from a qualitative standpoint.*

Proposed Procedure.

On the results of the foregoing investigation, the following procedure is proposed for the removal of all the phosphate present by means of zirconium oxychloride. The technique is simple; small volumes are involved, which obviate tedious evaporation after removal; there is complete removal, and a very slight excess of zirconium, which minimises its interference in later stages of the analysis. Moreover, all filtration difficulties due to colloidal formation are overcome:—

Transfer the solution to a boiling tube (small size), and adjust the volume so that it approximately half-fills the tube. Add 2 g. of ammonium chloride,

add a 10 per cent. solution of zirconyl chloride, a few drops at a time, without stirring or shaking. Allow the precipitate formed on addition of each few drops of the zirconyl chloride solution to settle before further addition. Continue adding until addition of a few drops to the clear supernatant solution causes no precipitation. Heat the mixture to boiling and filter hot. Treat the filtrate from this precipitation by the same procedure that would have been followed had phosphate been absent.

NOTE.—If the precipitate becomes too bulky before the end-point is reached. or does not completely settle, boil the mixture for one minute, and then allow to stand. It will now settle in much smaller bulk. (If the mixture after boiling is milky, and does not settle after 10 minutes, excess zirconyl chloride has definitely been added. Heat the mixture to boiling and filter hot.)

Excess Reagent.—Excess zirconium is precipitated with the iron-aluminium group, and will follow the iron. The small excess can be ignored, as it will cause no difficulty in the tests for that element by the usual ferro-cyanide or thiocyanate methods. If, however, it is desired to isolate the excess zirconium, the following procedure can be adopted:—

To the filtrate obtained after removal of the phosphate, add ammonium hydroxide till alkaline. This precipitates iron, aluminium, and chromium, if present, and excess zirconium, as hydroxides. Boil to complete precipitation. Filter and wash precipitate. The filtrate contains the metals of Groups 4–5. Reserve.

Ppt. Fe $(OH)_3$—red; Cr $(OH)_3$—green; Al $(OH)_3$—white; $Zr(OH)_4$—white. Wash into a boiling tube with cold water. Add carefully to the cold mixture Na_2O_2 in separate small quantities until the mixture is strongly alkaline. Heat *gently* till reaction ceases (no further evolution of gas). Filter and wash residue remaining on the paper.

RESIDUE:—$Fe(OH)_3$. Dissolve in a little warm HCl and test solution for iron.	FILTRATE $NaAlO_2$, Na_2CrO_4, Na_2ZrO_3. Boil for 2 minutes, filter, and wash residue.	
	RESIDUE:—$Zr(OH)_4$.	FILTRATE $NaAlO_2$, Na_2CrO_4. Separate and detect by the usual methods.

Loss of Ions.—In both the tin and ferric chloride methods, there is a considerable loss of ions (1). The Curtman procedure has also this serious disadvantage (2). There is no serious loss, however, by the Pittmann procedure. The loss of ions by the proposed modified procedure was now examined. Iron, nickel, barium, and magnesium were taken as representative of the metals of later groups, and the loss of these metals was investigated as follows:—One milligram of each ion was mixed separately with 140 mg. of phosphate, and the phosphate was removed by the proposed procedure. The filtrate was then tested for the ion that was present before removal of the phosphate.

The results showed that there is practically no loss of ions in removal by the proposed procedure. One milligram of each of the metallic ions, Fe, Ni, Ba, Mg, could easily be detected after removal of 140 mg. of phosphate.

Using the proposed procedure, the following mixtures of salts were analysed, and in each case the metallic ions were recognised:—(*a*) Potash alum and potassium phosphate; (*b*) ferric chloride and potassium phosphate; (*c*) chromium sulphate and potassium phosphate; (*d*) aluminium sulphate, ferrous sulphate, and potassium phosphate; (*e*) chromium sulphate, ferric chloride, and potassium phosphate; (*f*) aluminium sulphate, chromium chloride, and potassium phosphate; (*g*) ferric chloride, nickel sulphate, and potassium phosphate; (*h*) zinc carbonate and sodium phosphate; (*i*) barium chloride and potassium phosphate.

Summary.

The Pittmann procedure for the removal of phosphate by means of zirconium oxychloride has been examined. It has been found to have many disadvantages. The causes of these have been studied, and a new procedure has been proposed in which the difficulties of the Pittmann procedure are overcome. It has been shown that by this proposed procedure the phosphate is completely removed in an easily filterable form, with practically no loss of other ions. The technique is simple. Small volumes are involved, which obviate tedious evaporation after removal. The very slight excess of reagent by this procedure does not interfere in the detection of the metals of later groups, and can be ignored. However, a scheme is suggested by which it can be isolated from the iron-aluminium group, if desired. As a rule the whole operation of removal can be carried out in less than 15 minutes, whereas by the Pittmann procedure it takes approximately 2 hours. From the standpoint of ease of technique and rapidity of execution, the proposed procedure is an advance on the methods used heretofore.

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NOTE ON THE ANALYSIS OF AN IRISH PHOSPHATE ORE.

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Price One Shilling.

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A phosphate deposit occurs in a river valley near Doolin, in the west of County Clare, in what is classified, according to the general geological maps, as the limestone area. It is a hard rock phosphate, and from its mode of occurrence it may be classified as a sedimentary deposit formed by replacement of limestone. It is dark grey, almost black in appearance, and occurs as a fairly uniform bed 2 ft. 6 in. to 3 ft. in thickness, with an overburden of variable composition. This overburden consists in some places of light top-soil with 5 ft. to 7 ft. of shale, and in others of 5 ft. to 7 ft. of soil and clay with 2 ft. 9 in. of shale. Below the phosphate bed comes a further bed of shale 2 ft. 9 in. in thickness which is underlain by limestone.

A natural phosphate is of importance as a potential source of raw material for the manufacture of superphosphate to be used as a fertiliser. The chief constituent of such a phosphate is phosphoric acid (P_2O_5) or tribasic phosphate of lime ($Ca_3P_2O_8$), which is the form in which it generally occurs. Unfortunately, other constituents are almost invariably present, and these may be either diluents or actively disadvantageous, either in interfering with the manufacture of the superphosphate, or in lowering the quality of the product obtained.

Let us consider in order of importance the determination of the various constituents in a sample of the ore under investigation.

Phosphoric Acid (P_2O_5). The simplest method used for the estimation of total phosphate is the citro-magnesia method, which is purely gravimetric. This method is favoured in spite of its admitted defects because of its ease of manipulation, and also as it is comparatively speedy. It consists of precipitation of the phosphate as magnesium ammonium phosphate ($Mg \cdot NH_4 \cdot PO_4$) in the presence of ammonium citrate solution, which inhibits the co-precipitation of iron and alumina with the phosphate, and keeps the calcium in solution.

In other methods the phosphate is first precipitated as phospho-molybdate, and estimated, either volumetrically with standard alkali, or gravimetrically as magnesium ammonium phosphate, which on ignition is converted to magnesium pyrophosphate ($Mg_2P_2O_7$). The principle of these latter methods is the same, although the conditions of precipitation vary for different methods.

In his publication—"Analyse des Phosphates Naturels"—Paul Gavelle gives a comparative study of the citro-magnesia method and Jorgensen's phospho-molybdate method (1), and applies the two to the analysis of a Moroccan phosphate, and also to the analysis of a solution containing a known quantity of P_2O_5. The conclusions drawn from this comparative study are as follows:—

1. The citro-magnesia method applied to the analysis of phosphate cannot furnish, except accidentally, a strictly accurate content of P_2O_5. It admits of two causes of error in opposite directions:

(*a*) The solubility of ammonium magnesium phosphate in citrate of ammonia tends to make the results in P_2O_5 too low.

(*b*) The carrying off of lime in ammonium magnesium phosphate tends to produce too high results in P_2O_5.

Generally these two errors do not counterbalance each other. The extent of these errors varies, not only according to the composition of the phosphate analysed, but also according to the details of the mode of operation, which always varies from one chemist to another.

When this method is correctly followed the total of the two errors is slight, being about $+0{\cdot}3$ to $0{\cdot}4$ in the determination of P_2O_5 in a phosphate containing about 34 per cent. of P_2O_5, such as Morocco phosphate. This method, although sufficient for a manufacturer's purpose, cannot be accepted in the case of arbitrational analysis.

(2) The Jorgensen method, correctly carried out, gives contents of P_2O_5 which are scientifically accurate. The method of precipitation of the phospho-molybdate of ammonia prescribed by the author might with advantage be slightly modified. In our opinion the mineral solution should be heated to 50° C. before the solution of molybdate of ammonia is added.

Lundell and Hoffman in 1937 accepted the citro-magnesia method provided that two precipitations were effected. In estimating the P_2O_5 content of the sample, both the citro-magnesia method and Jorgensen's phospho-molybdate method were used.

The following are details of the citro-magnesia method in general use:—

Weigh 5 g. of the dried finely-ground sample into a 350 c.c. conical flask fitted with a small funnel (to prevent spitting). Add 50 c.c. of concentrated hydrochloric acid and 5 c.c. of concentrated nitric acid, and when effervescence ceases, boil gently for 30 min. Dilute with distilled water and filter into a 500 c.c. graduated flask, wash flask and filter well with hot distilled water. Cool, and make up to the mark with distilled water. Take 100 c.c. (equivalent to 1 g. of phosphate) for each estimation. Add ammonium hydroxide until a precipitate appears, and add immediately 75–125 c.c. of ammonium citrate solution according to the phosphate under examination. Next add 75–125 c.c. of magnesia mixture slowly and with constant stirring. Place on stirring machine, stir for half an hour, and allow to stand overnight. Filter through platinum gooch crucibles packed with asbestos, washing precipitate well with

3 per cent. ammonia solution until free from chlorides, and after drying ignite in a muffle furnace at 950–1000° C. The precipitate is weighed as $Mg_2P_2O_7$, and calculated as P_2O_5 or $Ca_3(PO_4)_2$ as required.

	Weight of $Mg_2P_2O_7$.	P_2O_5 Per cent.	$Ca_3(PO_4)_2$ Per cent.
Jorgensen Method.	0·185	23·60	51·56
	0·184	23·46	51·28
	0·185	23·60	51·56
	0·184	23·46	51·28
	Average	23·53	51·42
Citro-magnesia Method.	0·372	23·72	51·82
	0·373	23·79	51·96
	0·371	23·66	51 68
	0·372	23·72	51·82
	Average	23·72	51·82

Iron and Alumina. The iron in Clare phosphate is present in at least two forms, as oxide combined with the phosphate, and as sulphide (pyrites), the latter occurring as sporadic particles of varying size, some visible to the naked eye, others visible only under the microscope. There is also a further small amount of iron insoluble in acid, and soluble only on fusion, but this is an almost negligible quantity.

It is impossible to reproduce in the laboratory the actual conditions of mixing which occur in the manufacture of superphosphate with sulphuric acid as the solvent; consequently it is difficult to devise an absolute laboratory method for determining the amounts of iron and alumina which will react during the mixing, i.e. soluble iron and alumina. The most important factor governing these estimations seems to be the choice of a suitable acid solvent.

Lundell and Hoffman (2) recommend attack with constant boiling hydrochloric acid (1 : 1), and regard nitric acid as an unsatisfactory solvent, as it "has the disadvantage of dissolving pyrite, which is not wanted, and leaving haematite, which is." Aqua regia usually causes practically complete solution, and undoubtedly gives more "active iron and alumina, particularly iron, than is fair." In their routine method the iron and alumina are precipitated together as phosphates, the iron content is determined separately, and the alumina estimated by difference. By a second method (3) devised by the same analysts iron and calcium are removed before aluminium is precipitated as phosphate.

Owing to the variable results obtained for soluble alumina by these latter methods, concentrated hydrochloric acid was finally chosen as solvent for the phosphate. The iron and alumina were precipitated combined as phosphates

at pH (3·2–3·4) after the method of G. J. Austin (4). Therefore the results given for "soluble" iron and alumina are the amounts of these constituents soluble in concentrated hydrochloric acid, which acid is particularly suited to Austin's method of estimation.

For each estimation 5 g. of the sample were evaporated to dryness with 50 c.c. of concentrated hydrochloric acid, the residue taken up with 25 c.c. of the same acid, diluted to 100 c.c. with hot distilled water, and heated on the water-bath for 15 min., then filtered into a graduated 500 c.c. flask, the residue being washed well with hot distilled water. The flask and contents were cooled to room temperature, and the volume adjusted to the 500 c.c. graduation with distilled water.

The soluble iron is determined in 100 c.c. of the solution (representing 1 g. of the phosphate), using the method given in the Bureau of Standards pamphlet referred to previously. The iron and alumina are estimated together as phosphates, as previously stated, and the alumina determined by difference.

Using this method it was found that the phosphates were very easily filtered without the aid of filter-paper pulp, which on ignition tends to reduce the phosphates. Zinc, which appears to be the chief interfering element, was present in such small quantities that it was deemed unnecessary to reprecipitate the phosphates to avoid their contamination by this element.

Austin recommends the use of hot N/100 acetic acid as a wash solution, as "washing with ammonium acetate or nitrate solution tends to give low results owing to hydrolysis, whilst washing with ammonium phosphate solution tends to give too high results owing to adsorption of P_2O_5."

Results obtained:—

1st titration	...	4·95 c.c. $KMnO_4$	= 0 0123 g. Fe_2O_3	Mean = 0·0121 g.
2nd ”	...	4·85 ” ”	= 0 0120 ” ”	
3rd ”	...	4·90 ” ”	= 0 0121 ” ”	
4th ”	...	4·90 ” ”	= 0 0121 ” ”	

(1 c.c. KMnO solution = 0 00247 g. Fe_2O_3 or 0 00467 g. $FePO_4$.)
Soluble Iron = 1·21 per cent. Fe_2O_3.

Weights of combined iron and alumina phosphates.

No. 1 = 0·041 g.	Mean = 0·0405 g.
No. 2 = 0 040 ”	
No. 3 = 0·042 ”	
No. 4 = 0·039 ”	

($AlPO_4 \times 0{\cdot}418 = Al_2O_3$.)

Weight of $FePO_4$ equivalent to 0·0121 g. Fe_2O_3 = 0·023 g.
Weight of $AlPO_4$ = (0·0405–0·023) = 0·0195 g., equivalent to 0·0082 g. Al_2O_3.
Soluble Alumina = 0·82 per cent. Al_2O_3.

The figure obtained for soluble iron, using concentrated hydrochloric acid as solvent, agreed almost exactly with that obtained by solution in constant boiling hydrochloric acid, and also with that obtained when the mixed phosphates were dissolved in hydrochloric acid, and the iron was determined volumetrically.

The residue insoluble in concentrated hydrochloric acid on fusion with (Na_2CO_3–K_2CO_3) yielded an amount of iron equivalent to 1·85 per cent. Fe_2O_3. This includes the iron which is insoluble in aqua regia (0·07 per cent. Fe_2O_3). Addition of the two amounts, soluble and insoluble, gives a figure for total iron equal to 3 06 per cent. Fe_2O_3. The insoluble residue also yielded 0·18 per cent. Al_2O_3, and was estimated in a manner similar to that used for soluble alumina, with the addition of 5 c.c. of molar potassium dihydrogen phosphate at the stage indicated.

Results obtained:—

1st titration	... 7 6 c.c. $KMnO_4$	= 0·0187 g. Fe_2O_3		
2nd "	... 7 4 " "	= 0 0183 " "		Mean = 0 0185 g.
3rd "	... 7·5 " "	= 0·0185 " "		
4th "	... 7 5 " "	= 0 0185 " "		

Insoluble Iron = 1·85 per cent. Fe_2O_3.

Weights of combined iron and alumina phosphate.

No. 1 = 0 040 g.	
No. 2 = 0 038 "	Mean = 0 0395 g.
No. 3 = 0·041 "	
No. 4 = 0·039 "	

Weight of $FePO_4$ equivalent to 0·0185 g. Fe_2O_3 = 0 035 g.

Weight of $AlPO_4$ = (0 0395 – 0 035) = 0·0045 g.

Weight of Al_2O_3 = (0 0045 × 0 418) = 0·0018 g.

Insoluble Alumina = 0 18 per cent. Al_2O_3.

Total Iron and Alumina. The following method was used:—After evaporation to dryness of 5 g. of the sample with concentrated hydrochloric acid, as for soluble iron and alumina, take up with the same acid, and filter. Ignite residue, and fuse with (Na_2CO_3–K_2CO_3), take up the melt with dilute hydrochloric acid, filter, and wash well, adding the washings to the first filtrate. Remove silica by two evaporations to dryness with hydrochloric acid, take up the residue with 25 c.c. of concentrated hydrochloric acid, and proceed as for soluble iron and alumina.

The iron is determined in 100 c.c. portions of the solution, but only a 50 c.c. aliquot is taken for the alumina estimation, so that the weight of the combined phosphates approximates to that obtained for soluble iron and alumina.

Results obtained:—

1st titration	... 12·7 c.c. $KMnO_4$	= 0·031 g. Fe_2O_3	Mean = 0·031 g.
2nd „	... 12·5 „ „	= 0·031 „ „	
3rd „	... 12·65 „ „	= 0·031 „ „	
4th „	... 12·55 „ „	= 0·031 „ „	

Total Iron = 3·10 per cent. Fe_2O_3.

Weights of combined iron and alumina phosphates.

(50 c.c. aliquots = 0·5 g. of phosphate.)

No. 1	= 0 040 g.	Mean = 0·042 g.
No. 2	= 0·044 „	
No. 3	= 0·041 „	
No. 4	= 0·043 „	

Weight of $FePO_4$ equivalent to 0·031 g. Fe_2O_3 = 0·059 g.

Weight of $AlPO_4$ = (0·084–0·059) = 0·025 g. equivalent to 0 0105 g. Al_2O_3.

Total Alumina = 1·05 per cent. Al_2O_3.

The results obtained by addition of the quantities obtained by estimation in the soluble and insoluble portions are

3·06 per cent. Fe_2O_3 and 1·10 per cent. Al_2O_3.

Iron Sulphide (FeS_2). Deducting from the values obtained for total iron the amounts soluble in hydrochloric acid and insoluble in aqua regia, the residual percentage of iron is 1·81 per cent. Fe_2O_3, which is equivalent to 2·72 per cent. FeS_2.

Separate 1 g. portions of the phosphate were dissolved in aqua regia, and the sulphur estimated as barium sulphate in the usual fashion. After deduction of the amount of sulphur soluble in hydrochloric acid (0·075 per cent.), this gave an amount of sulphur equivalent to 2·69 per cent. FeS_2. These results agree pretty well with the figure (2·67 per cent. FeS_2) obtained by estimating the iron soluble in aqua regia after ignition of the original hydrochloric acid insoluble residue.

Carbonates. The carbonic acid content may be conveniently determined by difference in Schrotter's apparatus, using dilute hydrochloric acid to decompose the carbonates. Using this method it is difficult to be certain that all the carbon dioxide is driven off, and that nothing other than carbon dioxide has been lost. It is more accurate to collect the carbon dioxide by absorption in soda-lime, using the usual train of apparatus for drying and purifying the gas evolved. For each estimation 2 g. of the phosphate are placed in a decomposition flask and 100 c.c. of dilute hydrochloric acid added by means of a dropping funnel. After primary decomposition ceases the flask is heated just

to boiling point, while a slow stream of carbon-dioxide-free air is drawn through the apparatus.

Weights of Absorption Tube. Before.	After.	Weight of Carbon Dioxide absorbed.
51·678 g.	51·913 g.	0·235 g.
55·289 „	55·525 „	0·236 „
51·913 „	56·148 „	0·235 „
55·525 „	55·759 „	0·234 „
	Mean Weight	0·235 „

Carbon Dioxide = 11 75 per cent.

Fluoride. Fluorine is usually estimated by fusion of the phosphate with a mixture of sodium and potassium carbonates, and, after removal of interfering elements, by final precipitation as calcium fluoride or sulphate. The chief objection to final estimation as calcium fluoride appears to be the solubility of calcium fluoride in water, and the difficulty in filtering the precipitate. The fluorine is preferably precipitated as lead chlorofluoride (5), which has a much higher molecular weight than calcium fluoride. Errors due to solubility of the lead chlorofluoride are greatly reduced by the use of a saturated solution of lead chlorofluoride for the greater part of the washing of the precipitate.

For each estimation 1 g. of phosphate is fused with K_2CO_3, the melt leached with hot water, filtered, and well washed. The last traces of silica are removed by the addition of zinc oxide in nitric acid (1 g. ZnO in 30 c.c. N. HNO_3). The fluoride is precipitated by the addition of a large excess of lead chloride and allowed to stand overnight. It is filtered through a weighed Gooch crucible, washed once with alcohol, then four or five times with saturated lead chlorofluoride solution, and finally with cold water, and dried at 120–150° C.

Weight of crucible.	Weight of crucible and precipitate.	Weight of lead chlorofluoride.
17·930 g.	18·210 g.	0 280 g.
18·680 „	18·950 „	0·270 „

Fluorine = 2 per cent.

Silica. In the estimation of this element the method used was that of Berzelius (6) modified by Hoffman and Lundell.

1 g. of the phosphate is intimately mixed with 5 g. of fusion mixture (Na_2CO_3–K_2CO_3) covered with a further 1 g. of the mixture, fused at dull red heat, and the melt extracted with hot water.

The silica is separated in three stages, first by boiling with 2 per cent. sodium carbonate solution. The silicious residue is retained, and the filtrate

treated with zinc nitrate (1 g. ZnO in 30 c.c. N. HNO_3), boiled, and filtered from a second silicious residue. The new filtrate is rendered slightly acid with nitric acid, and to it is added a reagent (made by dissolving 1 g. ZnO, 2 g. $(NH_4)_2CO_3$ in 20 c.c. H_2O containing 2 c.c. NH_4OH (conc)). The mixture is boiled free from ammonia, and the volume adjusted to about 100 c.c. The third silicious residue is filtered off, combined with the two previous ones, evaporated to dryness with hydrochloric acid, washed with water containing the same acid (20 : 1), ignited, and weighed.

Weights of SiO_2, 1st estimation = 0·108 g.
2nd „ = 0 107 „
Percentage of SiO_2 = 10·75 per cent.

Calcium. The solution for estimation of total lime is prepared as in the method for total iron and alumina given earlier in this paper—50 c.c. aliquots (equivalent to 0·5 g. of phosphate) are treated as in the method given in the Bureau of Standards leaflet (3).

Weights of CaO obtained:—

1st estimation	=	0·226 g.
2nd „	=	0·225 „
3rd „	=	0·225 „
4th „	=	0·224 „

Percentage of lime (CaO) = 45·00.

"The method gives results that are comparable with those obtained by the ordinary oxalate method in rock analysis. Because of the solubility of calcium oxalate a little is unprecipitated in both methods, but the total amount need not exceed 0·5 mg. (0·1 per cent. CaO in a 0 5 g. sample in double precipitations). In the accurate analysis of minerals this is subsequently recovered by treatment of the weighed magnesium pyrophosphate; in the analysis of phosphate rock this refinement is not necessary.

The treatment with iron, ammonium hydroxide, and bromine serves quite satisfactorily for the removal of manganese and residual phosphoric acid, and numerous tests have shown that usually less than 0·2 mg. of CaO is carried down with the precipitate."

Zinc and Manganese. The solution for estimation is prepared as for total iron and alumina. The zinc is separated as sulphide at pH (4·6–4·8), and the manganese as oxide, using G. J. Austin's method of estimation (4). Aliquots equivalent to 2 g. of phosphate were used for each estimation, so that fairly appreciable weights of precipitate could be obtained. Both the sulphide and oxide are dissolved in the least possible quantity of 50 per cent. hydrochloric acid, precipitated as ammonium phosphates, and estimated finally as pyrophosphates (7).

Weights of zinc pyrophosphate.	Weight of zinc oxide.
0·015 g.	0·008 g.
0·011 „	0·006 „

(i.e. for 2 g. of phosphate.)

Percentage of ZnO = **0 35.**

Weights of manganese pyrophosphate.	Weight of MnO.
0·020 g.	0·010 g.
0·016 „	0 008 „

(i.e. for 2 g. of phosphate.)

Percentage of MnO = **0·45.**

Iodine. Two methods were used for the estimation of iodine. The first is that (8) in present use by Dr. Mason at University College, Cork, and is known as the "open-ashing" method. The second method is the Trevorrow and Fashena modification of Leipert's technique (9). In both these methods the iodine is estimated finally by titration with N/500 sodium thiosulphate, using starch as indicator, and the reagents are tested for freedom from iodine by running "blank" estimations in parallel.

Duplicate results were :—

"Open-ashing" Method.	Acid "wet combustion" Method.
100 γ per 100 g.	174 γ per 100 g.
	310 γ „ „ „

The results given for the second method are the most reasonable of four estimations, the other two results being excessive, probably due to adsorption of iodine in the laboratory. The results obtained by the first method being the more reliable are given in the table.

Arsenic. Arsenic is estimated by the usual Marsh method, by comparison of the stain produced with a standard. Two estimations were made, of which the mean result was 0·0001 per cent.

Organic Matter. Although this constituent was not determined in the particular sample analysed, it is to be expected that such a deposit should contain a certain amount of organic matter. In the absence of other metallic and acidic radicals, the amount of organic matter present may be taken as being equivalent to the undetermined portion of the analysis.

In all, twelve samples of the same deposit have been analysed for calcium phosphate content. The several results obtained varied from 49·32 per cent. to 51·90 per cent., and indicate comparative uniformity of the deposit. The table shows complete analysis of one such sample.

This was a macro-analysis, the micro-estimation of iodine and arsenic being introduced merely as points of interest. The sample was ground to pass completely through a 90 I.M.M. sieve, and dried for three hours at 100° C.

		Per cent.
Phosphoric Acid	(P_2O_5)	23·53 equivalent to 51·42% $Ca_3P_2O_8$.
Lime	(CaO)	45·00
Iron Oxide	(Fe_2O_3)	1·28
Alumina	(Al_2O_3)	1·05
Pyrites	(FeS_2)	2·70
Silica	(SiO_2)	10·75
Carbonic Acid	(CO_2)	11·75
Fluorine ...	...	2·00
Zinc	(ZnO)	0·35
Manganese	(MnO)	0·45
Nitrogen ...	...	0·22
Sulphuric Acid	(SO_3)	0·20
Undetermined		0·72
	Total	100·00
Loss on Ignition	...	10·65
Soluble Iron ...	...	1·21
Soluble Alumina	...	0·82
Arsenic ...	...	0·0001
Iodine ...	...	100 γ per 100 g.

This ore does not readily respond to treatment with sulphuric acid, and it has not been possible to render it as completely soluble as desired. On treatment with less than the theoretical amount of acid required, a low grade superphosphate with a high percentage of insoluble phosphate and of only moderate drillability is obtained.

The only minor constituents present in excessive quantities in the ore are carbonate and silica, and, chemically, these should not cause serious drawbacks. A feasible theory for the failure to render this phosphate soluble in a satisfactory manner is that these minor constituents are so distributed through the mass of the phosphate as to form a protective sheath over the tricalcium phosphate and to prevent the sulphuric acid from interacting with it.

It would not be possible in such a paper as this to consider the various reactions incidental to the manufacture of superphosphate, and for details of these reactions reference should be made to specialized literature on the subject. It is sufficient to state that the primary reaction is between the tricalcium phosphate and sulphuric acid with the formation of monocalcium phosphate and gypsum, and that the degree of solubility of the phosphate, and its

drillability, depend to a large extent on the amounts of the minor constituents present and the forms in which they occur.

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SALMON OF THE RIVER ERNE.

RESULTS OF THE EXAMINATION OF A SMALL COLLECTION OF SCALES AND DATA.

By ARTHUR E. J. WENT,
Department of Agriculture, Fisheries Branch, Dublin.

Price One Shilling.

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RESULTS OF THE EXAMINATION OF A SMALL COLLECTION OF SCALES AND DATA.

By ARTHUR E. J. WENT,
Department of Agriculture, Fisheries Branch, Dublin.

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With a valuable fish like the salmon it is not possible to purchase large samples for examination in the laboratory, as can be done with the herring and other less expensive fish. Recourse has, therefore, to be had to other methods of obtaining material. It has been found satisfactory to arrange with the manager of a commercial salmon fishery on the river which it is desired to investigate to collect samples of scales, together with particulars of the measurements (weight to nearest 2 oz., length to nearest 1/10th inch), the sex, the place, and date of capture. Over a number of years collections of scales and measurements of salmon taken in various rivers throughout Ireland have been made with the intention of obtaining information on the age and growth of salmon of these rivers. A small collection of material was available from the River Erne, and, although it was obviously inadequate in many respects, it was considered of sufficient interest to be worked out and reported upon.

The proportion of fish sampled showed some fluctuation over the season, and in most cases the results have been weighted against the catch figures for 1928 in order that a more accurate idea of the stocks of the river could be obtained. The weighted figures have been given in Tables 1–4 and in suitable places in the text.

The River Erne and its Catchment Area.

The River Erne is 64 miles long, and the catchment area of the river and its tributaries covers approximately 1,690 square miles. There is a large number of lakes in this system, the most important of which are Lough Erne, Upper Lough Erne, Loughs Oughter and Gowna, all on the main river, and Loughs MacNean, Nilly, and Garadice, on the tributaries. The river rises in County Longford, flows for the major part of its length in a northerly or north-westerly direction, and finally empties into an estuary, about four miles long, which is an inlet of Donegal Bay. In the upper part of the catchment area there are hundreds of small lakes which contain coarse fish only. Salmon seldom, if ever, penetrate into them.

The main river from Lough Oughter, and the main tributaries flowing into the main watercourse from the west, run over rocks of the Lower Carboniferous Limestone, and in consequence these waters are alkaline in character. The Annalee River, the most important tributary of the River Erne, and the River Erne itself above Lough Oughter, drain areas of rocks of the Silurian and Ordivician periods, which contain little lime, and thence the waters of these rivers are neutral or slightly acid in character. On the east side of Lough Erne a number of small tributaries run over rocks of the Old Red Sandstone,

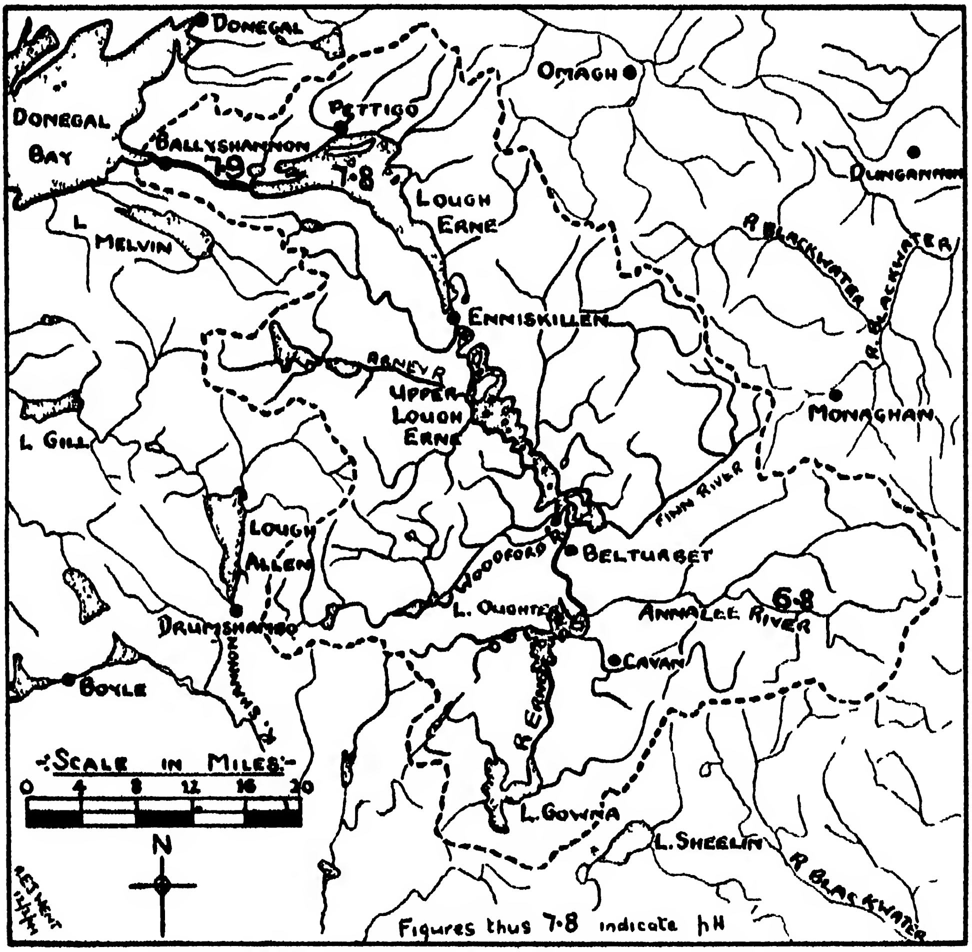

FIG. 1.—Catchment area of the R Erne, its lakes and tributaries (Based on the Ordnance Survey Map of Northern Ireland, by sanction of the Controller of H M. Stationery Office and the Ordnance Survey of Eire, by permission of the Minister for Finance of Ireland)

and in consequence these waters are acid in character. The relationship of the River Erne to its lakes and tributaries is represented in the form of a sketch-map in Fig. 1. On this figure a few pH figures have been inserted for the waters of particular localities.

Material.

The material used in this investigation consists of 421 satisfactory sets of scales and data collected from salmon captured in the tidal nets and the cribs below the bridge in the town of Ballyshannon, Co. Donegal, in the river in 1928, together with a few sets from the years 1921, 1926, and 1927. I am indebted to Mr. Thomas McD. Swan, of the Erne Fisheries, for collecting the material and furnishing me with confidential returns of the fishery for 1928. All the fish were measured from the tip of the snout to the fork of the tail to the nearest tenth of an inch.

Smolt Ages.

In the maiden fish, 346 fish (92·0 per cent.) migrated as two-year smolts, 18 fish (4·8 per cent.) as one-year smolts, and 12 fish (3·2 per cent.) as three-year smolts.

Age Groups.

The fish were classified into five groups of maiden or unspawned fish, and two groups of fish containing those which had previously spawned (i.e. with spawning marks on their scales). In the following table the fish are divided into their respective age groups :—

Winters in the sea.	1 +	2	2 +	3	3 +	With one S.M.	With two S.M.s
% of total	58·7	7·5	25·2	1 7	0 5	6·2	0·2

The maiden summer fish (1 +, 2 +, and 3 + winters) form 84·4 per cent. of the total numerically. Commercially the small summer fish, having relatively high average weights, form the most important age group, being approximately one-sixth heavier than the grilse, the next heaviest age group.

Confirmation of the fact that summer fish form a considerable part of the runs of salmon in this river can be obtained by consideration of the distribution in time of the catches of salmon (and grilse). In Fig. 2, which has been plotted from confidential figures kindly supplied by Mr. Thomas McD. Swan, the fortnightly percentages of the total catch for the year 1928 have been given. That similar conditions existed in other years can be shown by the fact that the appropriate curve for the year 1940,[1] which has been taken at random, shows a similar result.

[1] Up to and including the year 1933 the Erne Fisheries operated two draft nets in the tidal portion of the river and a set of cribs above the Falls of Assaroe in the town of Ballyshannon. In consequence of the judgment in the "Erne Case" in which it was decided that no several fishery existed in the tidal portion of the river, the fishery was thrown open to the public. From 1934 onwards a special local licence costing £40 for each draft net has been obtainable for fishing this river under the authority of the Fisheries (Tidal Waters) Act, 1934. Each licensee is bound to furnish a weekly return of his catches, and the curve for 1940 has been drawn up from the weekly returns of some thirteen licensees for that year. Since 1933 the Erne Fisheries have continued to operate the set of traps near the Falls of Assaroe.

As is usually the case, very few summer fish were captured in April; in May they formed just under half of the total catch, and thereafter they predominated (Table 1). May was the best month for the small spring fish, June for the grilse, small summer, and large spring fish, whilst most previously spawned fish were taken in July. The catches in June amounted to 64·6 per cent. of the total for 1928, and those of July to 24·0 per cent. (Table 2).

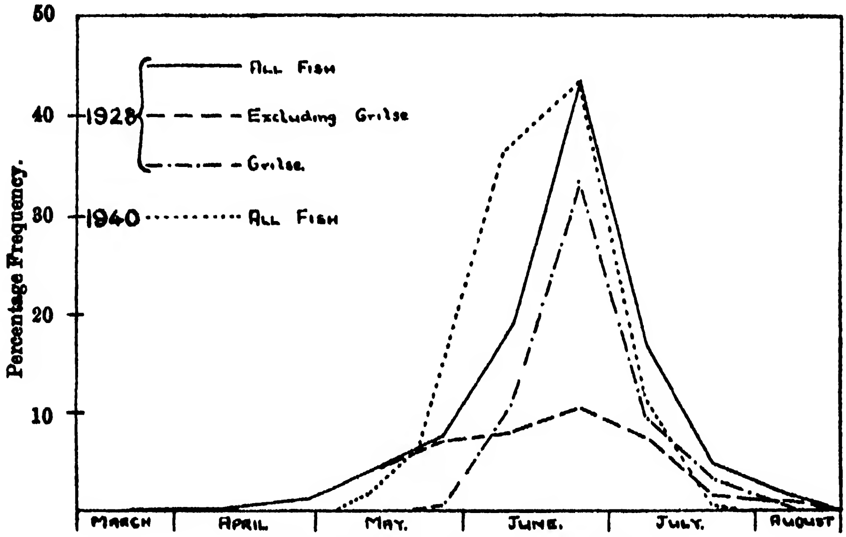

Fig 2—Distribution in time of the catches.

Forty-five fish, out of the total of 421 whose scales were examined, had spawned previously, as indicated by the presence of spawning marks on their scales. Two of these previously spawned fish had each spawned on two occasions, and were making their way into the river in anticipation of a third spawning. The determination of the age at first spawning is liable to some inaccuracy after the formation of the spawning mark and the return to the river as a clean fish, as erosion may remove traces of winter and even of summer growth. In Table 3 the age at first spawning has been correlated with the absence, which is the period spent feeding in the sea between what would be, but for the intervention of man, two successive spawnings. Owing to the difficulty of assessing the age at first spawning the figures in the table must be regarded as approximate. The summer fish generally show a tendency to return after a short or very long absence, while the spring fish return after a long absence. This is the condition which has been found in other investigations on the life history of salmon.

Size Distribution.

In Table 4 the estimated size distributions in each age group, as percentages of the total catch, have been given for two-inch class intervals. The curve

Table 1 Estimated Percentage[x] of each age group in each month's catch.

Period	Age Group						Total
	1+	2	2+	3	3+	With S.M.s	
22nd March to 30th April	-	48.0	7.7	21.2	-	23.1	100.0
May	2.8	32.3	43.1	7.0	-	14.8	100.0
June	68.7	4.4	22.9	1.3	0.2	2.5	100.0
1st July to 11th August	58.3	3.6	23.6	-	1.8	12.7	100.0
Total	58.7	7.5	25.2	1.7	0.5	6.4	100.0

Table 2 Estimated Percentage of total of each age group in each month.

Period	Age Group						Total
	1+	2	2+	3	3+	With S.M.s	
22nd March to 30th April	-	3.4	0.2	6.5	-	1.9	0.5
May	0.5	47.1	18.7	44.3	-	25.3	10.9
June	75.7	37.9	58.7	49.2	22.0	25.6	64.6
1st July to 11th August	23.8	11.6	22.4	-	78.0	47.2	24.0
Total	100.0	100.0	100.0	100.0	100.0	100.0	100.0

x For convenience the period 22nd March to 31st March has been included with April and the period 1st-11th August has been included with July in Tables 1 and 2.

Table 3 Previously spawned fish classified on absence.

Absence	Age at first spawning			Total
	1+ Winters	2 Winters	2+ Winters	
Short (less than complete year)	7	-	3	10
Long (complete year)	-	17	4	21
Very long (more than complete year)	6	-	6	12

Table 4 Frequency distribution of sizes in the different age groups.

Class Interval xx	Age Group						Total
	1+	2	2+	3	3+	With S.M.s	
16	1.2	-	-	-	-	-	1.2
18	0.6	-	-	-	-	-	0.6
20	9.4	-	-	-	-	-	9.4
22	25.8	-	-	-	-	-	25.8
24	18.8	0.1	-	-	-	0.1	19.0
26	2.9	0.4	-	-	-	0.2	3.5
28	-	2.0	1.5	-	-	0.4	3.9
30	-	3.0	9.2	-	-	0.6	12.8
32	-	1.6	9.6	-	-	0.6	11.8
34	-	0.4	3.6	0.2	-	1.3	5.5
36	-	-	1.0	0.4	0.5	1.6	3.5
38	-	-	0.3	0.3	-	1.3	1.9
40	-	-	-	0.4	-	0.2	0.6
42	-	-	-	0.4	-	0.1	0.5
Total	58.7	7.5	25.2	1.7	0.5	6.4	100.0

xx Class interval 16 etc. includes all fish having lengths between 15.95 and 17. 95 inches etc.

Table 5 Mean Condition Co-efficient (K) and Condition Factors (according to Corbett's scale) (C.F.) of the different age groups.

Age Group	Condition Co-efficient (K)	Condition Factor (C.F.)
1+ winters	1.17	42.2
2 winters	1.18	42.3
2+ winters	1.21	43.7
3 winters	1.25	44.8
3+ winters	1.11 (one only)	40.0
With S.M.s.	1.21	43.7
Total	1.18	42.6

Table 6 Showing divided migration and return. The following table gives the years in which the fish examined were hatched in percentages of the total catch for the year 1928.

Returned in 1928 as	Hatched in the year						Total
	1920	1922	1923	1924	1925	1926	
Grilse (1+ Winters)	-	-	-	1.7	55.8	1.2	58.7
Small Spring fish (2 Winters)	-	-	0.3	6.7	0.5	-	7.5
Small Summer fish (2+ Winters)	-	-	0.8	23.2	1.2	-	25.2
Large Spring fish (3 Winters)	-	0.1	1.5	0.1	-	-	1.7
Large Summer fish (3+ Winters)	-	-	0.5	-	-	-	0.5
With S.M.s †	0.1	4.1	1.2	0.9	0.1	0.1	6.4
Total	0.1	4.2	4.3	32.6	57.6	1.2	100.0

† Approximate values.

Table 7 Mean calculated lengths in inches in fresh water.

Smolt Age	Numbers	Lengths at end of			Mean Smolt Length
		1st Year	2nd Year	3rd Year	
1	18	3.1	-	-	5.2
2	332	2.1	5.0	-	5.6
3	12	2.0	4.3	5.8	5.8

Table 8 Estimated proportion of the different smolt types in each smolt class for maiden fish

Smolt Age	Type A		Type B	
	Number	Percentage	Number	Percentage
1	-	-	18	4.8
2	57	15.1	289	76.9
3	11	2.9	1	0.3

Table 9 Mean calculated lengths in inches in the sea in the various age groups.

Age Group	Numbers	Lengths at end of		
		1st Sea Winter	2nd Sea Winter	3rd Sea Winter
1+ Winters	100	18.3	-	-
2 Winters	87	19.0	30.9	-
2+ Winters	152	18.6	31.2	-
3 Winters	21	19.7	31.9	39.3

Table 10 Mean calculated lengths in inches in fresh water in the various smolt classes and types.

Smolt Age	Type A Smolts					Type B Smolts				
	Numbers	Lengths at end of			Smolt Lengths	Numbers	Lengths at end of			Smolt Lengths
		1st Year	2nd Year	3rd Year			1st Year	2nd Year	3rd Year	
1	-	-	-	-	-	18	3.1	-	-	5.2
2	57	2.3	5.7	-	5.7	289	2.0	4.8	-	5.5
3	11	2.0	4.3	5.8	5.8	1	1.7	4.0	5.3	5.8

showing the size distributions has two maxima at 22 and 30 inches, respectively, which are produced by the overwhelming abundance of the grilse and small summer fish, as can be seen from Fig. 3. Fish, the lengths of which were between 19·95 and 25·95 inches, comprised slightly more than half the total catch, whilst fish having lengths between 29·95 and 33·95 inches formed about one-quarter of the total catch.

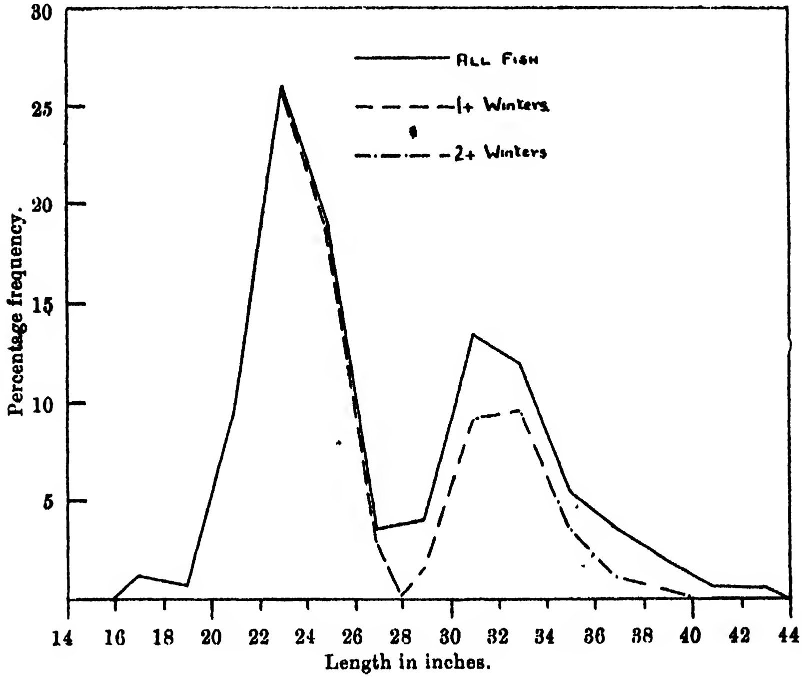

Fig. 3.—Estimated size distribution of the catches.

Average Sizes.

With a small collection of sets of scales and data such as were used in this investigation the monthly average weights and lengths of the different age groups are of little value, since in some cases only a few fish were sampled in any one month. In the following discussion the average sizes of the fish of the various age groups for the whole of the season have been given:—

(i) Grilse (1 + winters). Number examined = 105.

	lb.	inches.
Minimum	2·0	17·6
Average	5·4	23·2
Maximum	9·5	27·5

The smallest grilse was taken on the 31st May, together with another fish of the same length but weighing ½ lb. more, whilst the maximum weight and length were recorded on the 11th July. Out of 105 grilse, 2 had migrated as

one-year smolts, 100 (95·2 per cent.) as two-year smolts, and 3 had migrated as three-year smolts.

(ii) Small spring fish (2 winters). Number examined = 88.

	lb.	inches.
Minimum	5·5	24·0
Average	12·5	30 9
Maximum	18·75	35·8

The smallest fish of this age group was taken on the 19th May, whilst the maximum weight and length were recorded on the 4th May. Out of the 88 small spring fish examined, 6 had migrated as one-year smolts, 78 (88 8 per cent.) as two-year smolts, and 4 as three-year smolts.

(iii) Small summer fish (2 + winters). Number examined = 161.

	lb.	inches.
Minimum	9·0	28·0
Average	14·7	32·5
Maximum	26·0	37·8
	23·25	38·2

The smallest fish was taken on the 19th May. A weight of 26 lb. was recorded twice on the same day, namely, 2nd June, 1927, both fish having the same length. A longer but lighter fish, weighing only 23·25 lb. (length 38 2 inches), was captured on 24th May. Out of the 161 fish whose scales were examined, 9 had migrated as one-year smolts, 148 (91 8 per cent.) as two-year smolts, and 4 as three-year smolts.

(iv) Large spring fish (3 winters). Number examined = 21.

	lb.	inches.
Minimum	16·5	34·4
Average	27·9	39·3
Maximum	40·0	43·7

The smallest fish was taken on the 22nd May, and on the 31st May the largest fish was recorded. Out of the 21 sets of scales examined, one was taken from a fish which had migrated as a one-year smolt, 19 as two-year smolts, and one had migrated as a three-year smolt.

(v) Large summer fish (3 + winters).

Only one fish, weighing 21 lb., measuring 37·4 inches in length, and captured on the 4th June, was examined in this age group.

(vi) Previously spawned fish (with S.M.s). Number examined = 45.

This group comprises a heterogeneous collection of individuals having only

one common property, namely, the presence of one or more spawning marks on their scales.

	lb.	inches.
Minimum	7·0	24·0,
Average	19 7	35 2
Maximum	33 0	42 3

The smallest fish, captured on the 6th July, had spawned for the first time as a grilse, and had returned to fresh water for the second time after a period feeding in the sea of only a few months The largest fish was captured on the 9th May, and showed two spawning marks on its scales. Average weights and length in the case of previously spawned fish are, however, of little value, as the weight of a previously spawned fish will depend on at least three factors, namely, the age at first spawning, the period spent feeding in the sea between successive spawnings (absence habit), and the number of times a fish had spawned. It is usual to classify the previously spawned fish on their absence habit and the age at first spawning, but in this investigation the numbers of fish in this category (i.e. with S M.s) are too few to give significant results.

Condition Coefficients.

In a previous publication dealing with the salmon of the Ballisodare River (Went, 1941) the method of calculating the condition coefficient (K) and the condition factor (C.F.) or the relationship between weight and length was described. The mean condition coefficients (K) and condition factors (C.F.) have been given in Table 5. The mean condition coefficient rises as the time spent feeding in the sea increases. The high mean condition coefficient of the salmon of the River Erne is of the same order as that of the salmon of the Rivers Shannon and Ballisodare, although the small summer fish of the River Erne were superior in mean condition coefficients to those of the River Shannon.

Divided Migration and Return.

Table 6 gives the years in which the fish were hatched. The brood of one year, namely, that of 1925, was responsible for over half the catch in 1928, whilst the brood of 1924 accounted for one-third of the catch in 1928.

The Character of the Annuli in the Central Region of the Scales.

During the examination of the scales it became clear that the fish could be divided into two fairly distinct groups depending on the appearance of the annuli in the central region of the scale corresponding to freshwater or parr growth. In 10 per cent. or thereabouts of the fish the winter band was poorly defined. These differences were probably the results of life in different environments, and seemed worthy of note.

Calculated Lengths.

The lengths of every maiden or unspawned fish were calculated for the end of every year of life by the usual method, i.e. assuming that the growth of the scale was strictly proportional to that of the fish.

(*a*) *River Life.*

In Table 7 the mean lengths in inches of the different smolt classes have been given, and these are illustrated graphically in Fig. 4A. It will be seen that the fastest growing smolts migrated first, and that the mean smolt length increased as the age of the smolt rose.

In previous publications on the biology of the salmon of the Rivers Shannon, Ballisodare, and Owenduff (Ballycroy), it was stated that the fish of these rivers could be divided into two smolt types, called for convenience, Type A and Type B smolts. These were (1) Type A smolts, those fish which showed little or no growth in fresh water in the spring prior to migration as a smolt, and (2) Type B smolts, those fish which showed considerable amount of growth in fresh water in the spring prior to migration as a smolt.

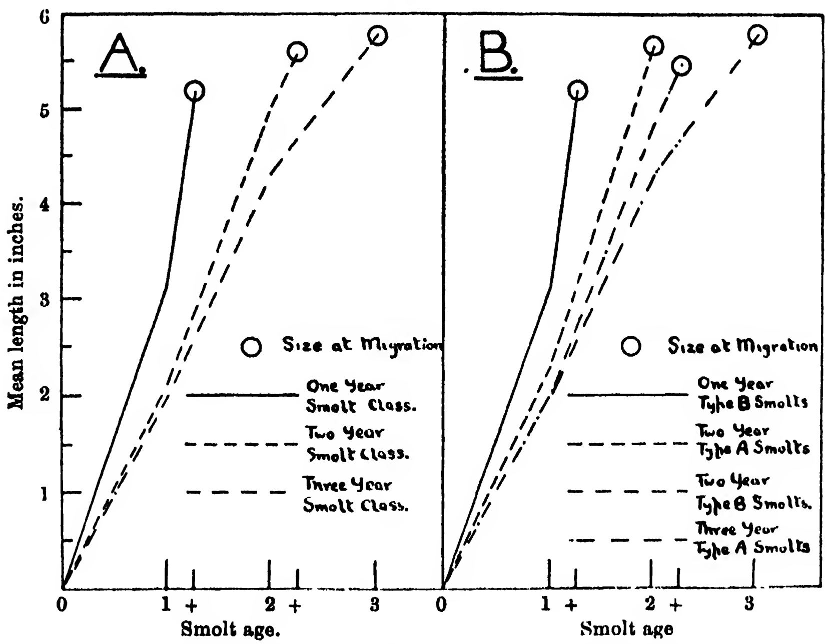

FIG. 4.—Growth in freshwater. A.—In the 1-, 2-, and 3-year smolt classes. B.—In the various smolt classes and types.

In the present material this division of the smolts into two groups was again carried out, and the results for the maiden fish are given in Table 8. The mean lengths in each of these groups have been calculated, and the results given in Table 10 are illustrated in Fig. 4B. In the two-year smolt class the

Type A smolts at the end of each year were longer than those of the Type B group. The material is too meagre, however, to draw any definite conclusions, but it can be noted that the results are of the same character as has already been described for other Irish rivers (Went, 1938, 1940, and 1941). The mean length of all the smolts was 5·6 inches, which is of the same order as that for the River Shannon (Went, 1940) and the Waterville River (unpublished data).

(*b*) *Sea Life.*

The calculated lengths for each year in the sea in each age group have been given in Table 9, and it will be seen that the growth rate in the large spring fish was greater than in any other age group.

Résumé.

(1) A brief description of the River Erne and the geology of its catchment area was given, Fig. 1.

(2) Material consisting of 421 satisfactory sets of scales from the River Erne nets and cribs was examined.

(3) Ninety-two per cent. of the fish had migrated as two-year smolts, whilst the remaining eight per cent. were one and three-year smolts in the ratios of 3 to 2 respectively.

(4) Almost eighty-four per cent. of the fish were summer fish (58·7 per cent. grilse and 25 2 per cent. small summer fish).

(5) The average sizes in the various age groups have been given. The mean condition coefficients of the salmon of this river are high, being of the same order as those for fish of the Rivers Shannon and Ballisodare (Went, 1938, 1940, and 1941).

(6) The lengths at the end of each year of life have been calculated. The fastest growing fish migrated first. As in the River Shannon salmon the smolts could be divided into two groups, depending on whether or not the fish had made growth in fresh water in the spring prior to migration as a smolt. Type A smolts of any smolt class have a greater mean length at the end of every year of life than Type B smolts of the same smolt class. In the sea the large spring fish had a greater average growth-rate than any other age group.

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THE

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[SEPARATE ISSUE.]

No. 2. JULY, 1942.

THE CHEMICAL CONSTITUENTS OF LICHENS FOUND IN IRELAND:

CLADONIA IMPEXA HARM.

BY T. W. BREADEN, M.SC., J. KEANE, PH.D., AND T. J. NOLAN, D.SC.,
University College, Dublin.

Price Sixpence.

No. 2.

THE CHEMICAL CONSTITUENTS OF LICHENS FOUND IN IRELAND.

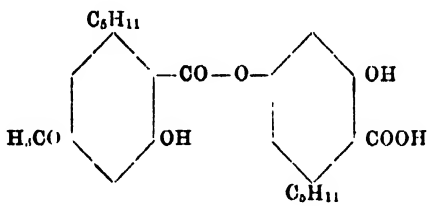
CLADONIA IMPEXA HARM.

BY T. W. BREADEN, M.Sc., J. KEANE, PH.D., AND T. J. NOLAN, D.Sc.,
University College, Dublin

[Read MAY 27, 1941. Published separately JULY 28, 1942.]

THERE appears to be no previous record of the occurrence in Ireland of the lichen *Cladonia impexa* Harm. It is not mentioned in the standard reference-Knowles, "The Lichens of Ireland." We have, however, found it growing in considerable quantities on peaty soil, amongst granite rocks on the west side of Killiney Hill, Co. Dublin. The specimens were identified for us by Mr. Mackenzie-Lamb, of the Natural History Section of the British Museum, to whom we wish to express our thanks. Asahina records (Acta Phytochimica, 1934, 8, 52) that the lichen shows a negative reaction to benzidine and p-phenylenediamine, indicating the absence of depsidones of the salazic acid type.

We have found the predominant constituent to be l-usnic acid We have also isolated perlatolic acid



previously found by Asahina and Fuzikawa (Ber., 1935, 68, 634) in the lichen *Parmelia cetrarioides*. There is also present a sterol giving a violet-red Liebermann reaction, changing to blue and then green. For the sterol we suggest the alternative formulae $C_{32}H_{50}O_4$ or $C_{34}H_{52}O_4$; the possibility that a mixture of sterols may be present is not excluded. The sugar-alcohol in the lichen is d-arabitol.

EXPERIMENTAL.

Ether Extract of Lichen.

700 g. of air-dried lichen were extracted with three successive lots of 5 l. of ether. The ether solution on evaporation gave 12·5 g. of a dark brown paste. This paste was stirred with 80 c.c. ether; on filtration a greenish brown

solution A and a residue B, which was a mixture of grey and yellow material, were obtained.

The solution A was extracted with successive portions of 50 c.c., 20 c.c., and 20 c.c., of 3 per cent. sodium carbonate solution. The alkaline extract foamed strongly on agitation and quickly set to a gel. It was acidified with dilute sulphuric acid, and extracted several times with ether; from this ether extract a brown gummy mass containing some crystals was obtained. This was boiled with 60/80 petrol, the solvent decanted from a gummy residue, and allowed to cool. The first material precipitated from the petrol was a gum, which was removed, and the mother liquors on standing deposited a crystalline product, which was purified by fractional crystallisation from 60/80 petrol. The purification was tedious, and eventually a crystalline product was obtained which had m.p. 104–106° C.

Analysis.

3·660 mg. gave 9 040 mg. CO_2, 2 350 mg. H_2O.
3 519 mg. gave 1·810 mg. AgI.
0·14 mg.—1 16 mg. camphor—21 0Δ.
Found C = 67 3; H = 7 1; OCH_3 = 6 8, M W. = 230.
$C_{21}H_{20}O_6 . OCH_3$ requires C = 67 5; H = 7 2; OCH_3 = 6 98;
M.W. = 444.

The substance is soluble in benzol, petrol, acetone, and alcohol. The alcohol solution is acid to litmus, and gives a red-brown colour with ferric chloride; reaction with bleaching powder is negative. The half value for the molecular weight indicates a depside. The formula, reactions, and melting point indicate that the material is perlatolic acid. This was confirmed by the products of hydrolysis, as obtained in the following experiment:—0·18 g. of the substance was heated at 60° C. with 4 c.c of 5 per cent. methyl alcoholic potash for 3 hours in a hydrogen atmosphere. From the reddish solution obtained the greater part of the alcohol was removed, the solution diluted with 25 c.c. water, and the whole saturated with carbon dioxide. On extraction with ether a product was obtained which was hydrolysed by boiling with 4 c.c aqueous potash; acidification and extraction with ether gave a material which on crystallisation from 60/80 petrol had m.p. 123–125° C. It was the p-methyl ether of olivetol carboxylic acid.

Analysis.

3·201 mg. gave 7·710 mg. CO_2, 2·150 mg. H_2O.
4·118 mg. gave 4·170 mg. AgI.
Found C = 65·6; H = 7·4; OCH_3 = 13 4.
$C_{12}H_{15}O_3 . OCH_3$ requires C = 65 5; H = 7 5; OCH_3 = 13·0.

The reddish solution referred to above was, after extraction with ether, acidified with dilute sulphuric acid, and extracted with ether. The product

recovered from the ether was crystallised from dilute methyl alcohol, and gave white needles m.p. 138–140° C., which were olivetol carboxylic acid.

Analysis.

3·490 mg. dried at 100° C. lost 0·150 mg.
3·340 mg. dried product gave 7·800 mg. CO_2; 2·100 mg. H_2O.
Found C = 63·7; H = 7·0.
$C_{12}H_{16}O_4$ requires C = 64·3; H = 7·1.
$C_{12}H_{16}O_4 . \frac{1}{2}H_2O$ requires H_2O = 4·0; Found 4·3.

The residue B referred to above, obtained by treatment of the lichen extract with a small amount of ether, was warmed with 30 c.c. chloroform; 0·7 g. of a cream-coloured powder C remained undissolved. The chloroform solution, on addition of alcohol, gave a precipitate of yellow needles, which, on recrystallisation from a mixture of alcohol and chloroform, had m.p. 205° C., and in chloroform solution was lævo-rotatory with a specific rotation of 485. It was l-usnic acid.

Analysis.

3 732 mg. gave 8 580 mg. CO_2; 1 520 mg. H_2O.
Found C = 62·9; H = 4·6.
$C_{18}H_{16}O_7$ requires C = 62 8; H = 4 6.

The powder C was treated with cold ethyl alcohol, in which it was partly soluble. The alcohol solution was evaporated to dryness, treated with methyl alcohol, which left behind a little usnic acid, and the methyl alcohol solution precipitated with water. The precipitate which still contained traces of usnic acid was warmed with chloroform to remove the latter, and was then crystallised from a small amount of ethyl alcohol, from which it separated in long white needles m.p. 245–250° C. The product, which is a sterol, is, in view of the unsatisfactory melting point, possibly a mixture of sterols. Though the preparation was repeated several times from different samples of lichen in no instance was a sharp melting point obtained. There was not enough material available to carry out a satisfactory chromatogammic separation.

Analysis.

(1) 3·340 mg. gave 9·450 mg. CO_2; 3·090 mg. H_2O.
(2) 3·720 mg. gave 10·610 mg. CO_2; 3·500 mg. H_2O.
(3) 3·698 mg. gave 10·500 mg. CO_2; 3 390 mg. H_2O.
(4) 0·664 mg.—7·040 mg. camphor—7·0°Δ M.W. = 498.
Found (1) C = 77·1; H = 10·27.
(2) C = 77·8; H = 10·4.
(3) C = 77·4; H = 10·2.
$C_{32}H_{50}O_4$ requires C = 77·1; H = 10·0 M.W. = 498.
$C_{33}H_{52}O_4$ requires C = 77·3; H = 10·1 M.W. = 512.

The substance gives with acetic anhydride and sulphuric acid a reddish violet colour which changes to blue and then green on standing. The analyses exclude the possibility of it being Zeorin.

The portion of the powder C insoluble in cold ethyl alcohol was boiled with alcohol until it gave no Liebermann reaction. It was then crystallised from aqueous acetone, from which it separated in small plates, m.p. 220° C. (with gas evolution). The substance is soluble in dilute sodium bicarbonate, gives a reddish brown colour with ferric chloride, but no reaction with bleaching powder. It contains methoxyl, but does not give the characteristic reaction of squamatic acid with sulphuric acid.

Acetone Extract of Lichen.

After extraction with ether the lichen was extracted with 5 l. of boiling acetone. The acetone solution on evaporation gave a somewhat gummy solid. This was extracted with water, and the aqueous solution evaporated to dryness; the material thus recovered was dissolved in ethyl alcohol, cleared with active charcoal, filtered, and the filtrate, after evaporation to a small bulk, treated with 10 volumes of dry ether. The material thus obtained was acetylated with acetic anhydride and sodium acetate, and the product obtained on pouring into water crystallised from ligroin, from which it separated in small plates, m.p. 72–74° C. A mixed melting point with d-arabitol pentacetate showed no depression.

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[SEPARATE ISSUE.]

No. 3. September, 1942.

ATMOSPHERIC POLLUTION IN DUBLIN DURING THE YEAR 1941.

By A. G. G. LEONARD, BRIDGET P. McVERRY,

and D. CROWLEY.

Price One Shilling.

No. 3.

ATMOSPHERIC POLLUTION IN DUBLIN DURING THE YEAR 1941.

By A. G. G. LEONARD, BRIDGET P. McVERRY, and D. CROWLEY.

[Read March 24. Published separately September 2, 1942.]

Work has been continued during 1941 at Leinster Lawn and the Albert Farm, Glasnevin.

Results obtained with Standard Gauges.

Details of the deposits during the year at Leinster Lawn and the Albert Farm are given in Tables 1 and 2 respectively.

Soluble solids deposited during the year at Leinster Lawn amounted to approximately 74 tons per square mile, and were about 1 6 times the amount at Glasnevin.

Insoluble solids at Leinster Lawn were deposited at the rate of 126 tons per square mile, and were 3 4 times the amount at Glasnevin.

The figure for insoluble matter at Glasnevin is 37 per cent. higher than that recorded in 1940, otherwise the deposits are not very different from those recorded in 1940.

Suspended Impurity.

The shortage of coal during 1941 has had a very marked effect on this impurity. A comparison of the figures for suspended impurity during 1941 with the average for the three years 1938, 1939, and 1940 is given in fig. 1a. From February, 1941, onwards there is a marked diminution in the degree of pollution as compared with previous years; actually the total pollution due to suspended impurity in 1941 was only one-half of the average for the preceding three years.

The number of "hazy days" (i.e. days upon which a shade number of 4 or more was recorded at any hour by the automatic filter) at Merrion Street in 1941 was 20, while the average number for the previous three years was 37.

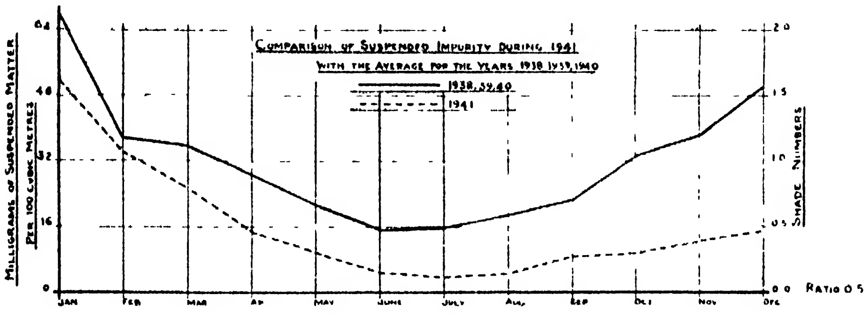
The following table gives the detailed results from February, 1938, when measurements were begun :—

Number of Hazy Days.

	Jan.	Feb.	Mar.	Oct.	Nov.	Dec.	Total.
1938...	—	4	7	3	7	9	30
1939...	9	3	1	7	3	9	32
1940...	17	8	7	3	9	6	50
1941...	11	4	3	0	1	1	20

The effect of the coal shortage is most marked in the last three months of 1941.

The same effect is to be noticed by a glance at the graphs for suspended impurity in fig. 1a.



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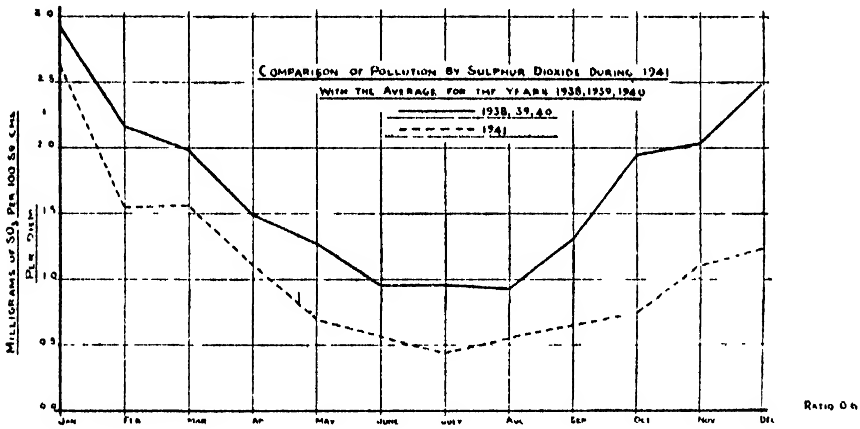

b

FIG. 1.

Suspended Impurity and Deaths from Respiratory Diseases.

The curves of moving averages for both of these are given in fig. 2. The maximum on the impurity curve in the past winter occurs at January 24th, 1942, and shows a depth of pollution of about the same order as the minimum prevailing during the summer months of 1939 and 1940. Since minima of pollution in the summer months correspond approximately to minima in the deaths from respiratory diseases, it may be taken that the low maximum of pollution in the past winter is of no significance as affecting the rise of deaths from respiratory diseases. It is to be noted, however, that the maximum of deaths from respiratory diseases during the past winter is the lowest recorded in the years during which this investigation has been carried out, a fact which is probably due to the decline in the contributory factor of suspended impurity.

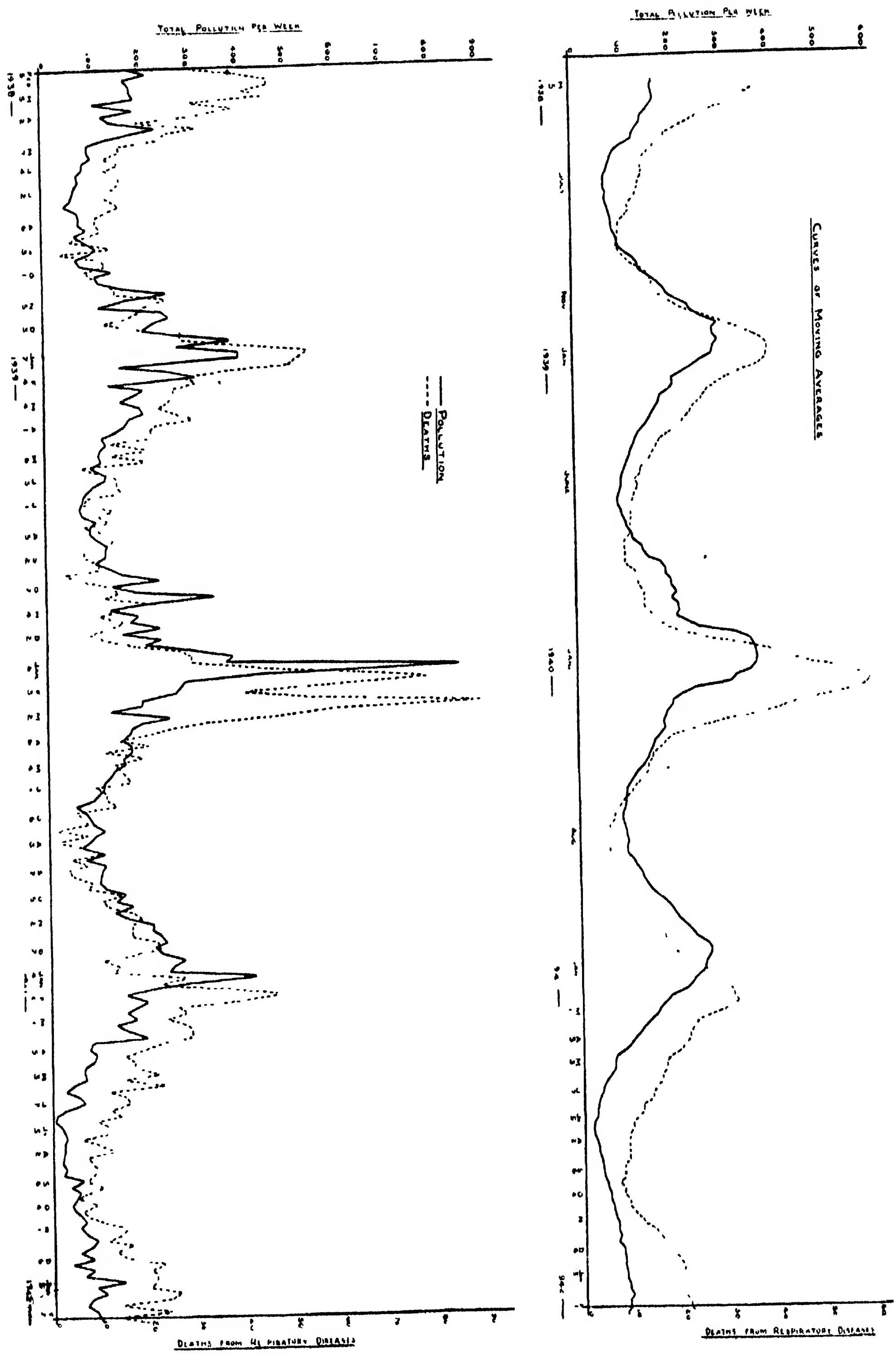
Total Pollution per Week
Deaths from Respiratory Diseases
Pollution
Deaths
Curves of Moving Averages
Total Pollution per Week
Deaths from Respiratory Diseases

Fig. 2.

Sulphur Dioxide.

Fig. 1b shows the monthly graph for sulphur dioxide at Leinster Lawn in 1941 determined by the lead peroxide method, compared with the average values for the three years 1938, '39, and '40. It may be seen that the curves are very similar in trend to those for suspended impurity over the same periods. The total pollution by sulphur dioxide in 1941 was 0 6 of the average of the previous three years. Fig. 3 shows the graphs for sulphur dioxide at the Albert

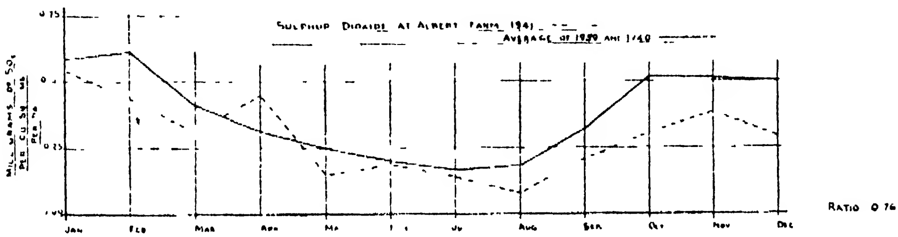

FIG 3

Farm during 1941, and the average for the years 1939 and 1940. Except for the month of April, the figures for 1941 lie below those for 1939–1940, the total for 1941 being 0 76 of the average of the previous two years.

Wind Velocity, Direction and Pollution by Suspended Impurity.

In our first paper on atmospheric pollution (Sci. Proc. R.D.S., 1939) some attempt was made to classify the above quantities. As the coal shortage has produced such a marked reduction in the intensity of suspended impurity during 1941 it has been decided to classify the observations obtained during the winter months of 1938, 1939, and 1940, which may be taken as representing years of normal coal consumption. We are indebted to Dr. H. H. Poole for suggesting the method of representation given in fig. 4.

The concentric circles represent depth of pollution increasing from the centre (0) to the outer circle (5). The dotted lines connect points representing the average values of the shade numbers recorded on the automatic filter at 9 a.m. for the wind directions and velocities (Beaufort, Nos. I to IV), indicated, as recorded by Mr. J. Gilmour at 9 a.m. at Trinity College. For calm (Beaufort, No. 0), the average shade was 5 0, as indicated by the darkening of the outer circle.

It is to be noticed that the winds N.N.W., N.W., and W.N.W. are on the whole most heavily polluted. The automatic filter is situated in the north wing of the Science Buildings, Upper Merrion Street, and such winds have passed over a large area of the city before measurement. So far as we are aware there

TABLE 1.—*Impurity Deposited from the Atmosphere at Leinster Lawn during the Year 1941.*

Grams per square Decametre (Metric Tons per Hundred Square Kilometres).

	Rainfall.	PH of rain-water.	*Insoluble Matter.*			*Soluble Matter.*		Total solids.	*Included in Soluble Matter.*			
	mm.		Tar.	Carbonaceous other than tar.	Ash.	Loss on ignition.	Ash.		Sulphates (SO_3).	Chlorine (Cl.).	Ammonia (NH_3).	Lime (CaO).
January	105	4·8	17	137	211	178	421	964	92	214	5	30
February	66	5·8	19	119	271	128	213	750	72	102	4	41
March	96	5·4	20	184	327	159	238	928	88	120	8	31
April	69	4·8	15	130	179	53	125	502	51	33	1	69
May	68	5·5	21	233	356	23	149	782	47	24	2	35
June	20	6·8	8	169	189	16	97	479	33	8	1	20
July	36	6·8	7	196	277	11	120	611	32	7	1	27
August	61	6·6	6	230	205	27	172	640	57	21	4	36
September	17	5·5	5	188	173	7	96	469	37	6	1	18
October	52	5·3	5	244	235	86	141	711	47	21	6	33
November	81	5·8	5	164	184	125	213	691	79	93	7	31
December	34	5·5	6	212	85	67	115	485	52	32	4	32
Mean Monthly	59		11 B	184 B	224 B	73 A	175 B	667 B	56 A	57	4	34
Summer Total	271		62	1146	1379	137	759	3483	237	99	10	205
Winter Total	434		72	1060	1313	743	1341	4529	430	582	34	198
Annual Total	705		134	2206	2692	880	2100	8012	667	681	44	403

TABLE 2.—*Impurity Deposited from the Atmosphere at Albert College, Glasnevin, during the Year 1941.*

Grams per Square Decametre (Metric Tons per Hundred Square Kilometres).

	Rainfall. mm.	PH of rain-water	*Insoluble Matter.* Tar.	Carbon-aceous other than tar.	Ash.	*Soluble Matter.* Loss on ignition.	Ash.	Total solids.	*Included in Soluble Matter.* Sulphates (SO_3).	Chlorine (Cl.).	Ammonia (NH_3).
January . .	64	4·95	4	21	20	131	146	322	63	94	1
February . .	44	4·92	3	23	40	126	72	264	35	65	3
March . . .	72	4·74	1	35	68	81	178	363	36	98	2
April . . .	66	5·28	2	32	52	67	84	237	38	43	7
May . . .	66	5·22	–	52	113	78	50	293	27	14	8
June . . .	22	6·78	–	123	67	96	69	355	21	11	22
July . . .	45	6·36	–	99	77	33	42	251	21	7	—
August . .	69	6·23	1	58	43	52	38	192	18	14	1
September .	22	5·94	2	102	141	34	39	318	29	8	2
October . .	46	5·79	3	61	49	40	50	203	25	17	2
November .	52	5·31	6	51	69	118	81	325	37	65	3
December .	36	5·11	1	30	24	40	40	135	18	21	4
Mean Monthly	50		2	57	64	75	74	272	31	38	5
Summer Total	290		5	466	493	360	322	1646	154	97	40
Winter Total	314		18	221	270	536	567	1612	214	360	15
Annual Total	604		23	687	763	896	889	3258	368	457	55

is, in this direction, no source of heavy pollution in proximity to the automatic filter, and the graphs, therefore, appear to give a fair representation of pollution in so far as wind direction is concerned.

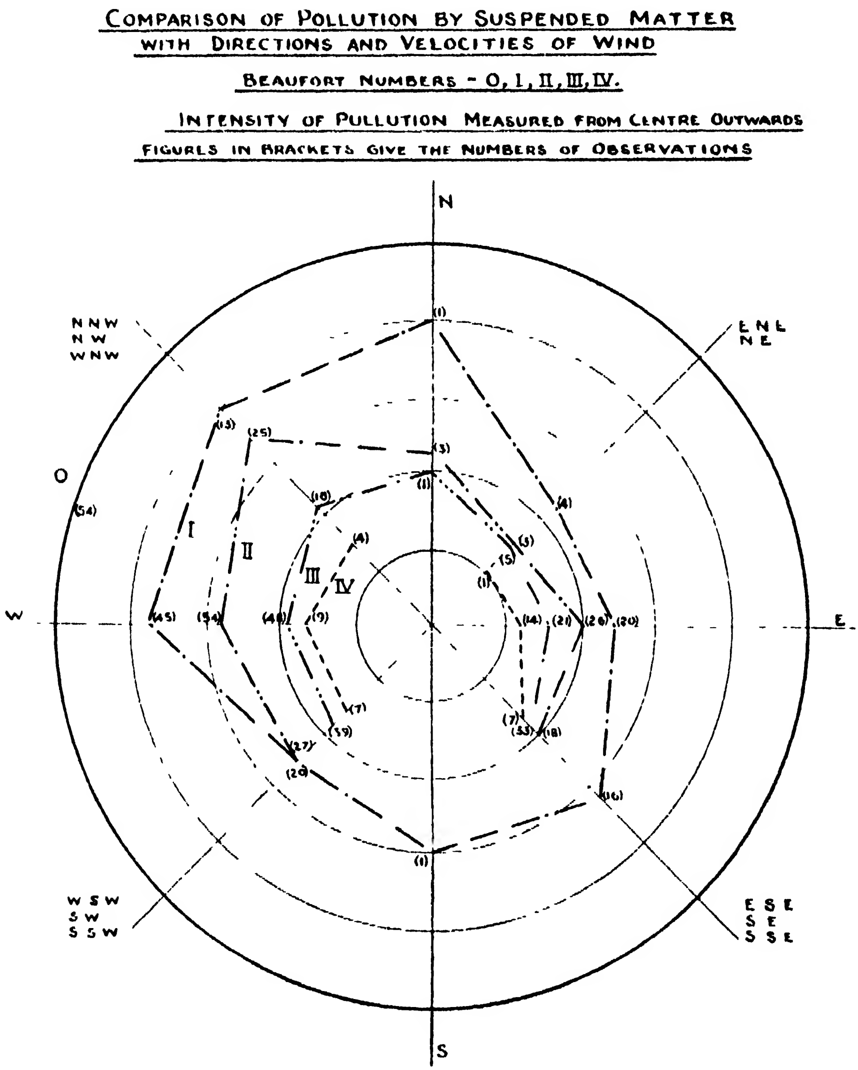

Fig 4.

Measurements of Sunlight.

Fig. 5 gives the records from January, 1941, to March, 1942. The loss of light at Merrion Street was considerable during the early part of 1941, falling to a much lower figure during the period May to June. The losses during the

last two periods on the graphs are the lowest so far recorded for those periods during the three years for which records have been made.

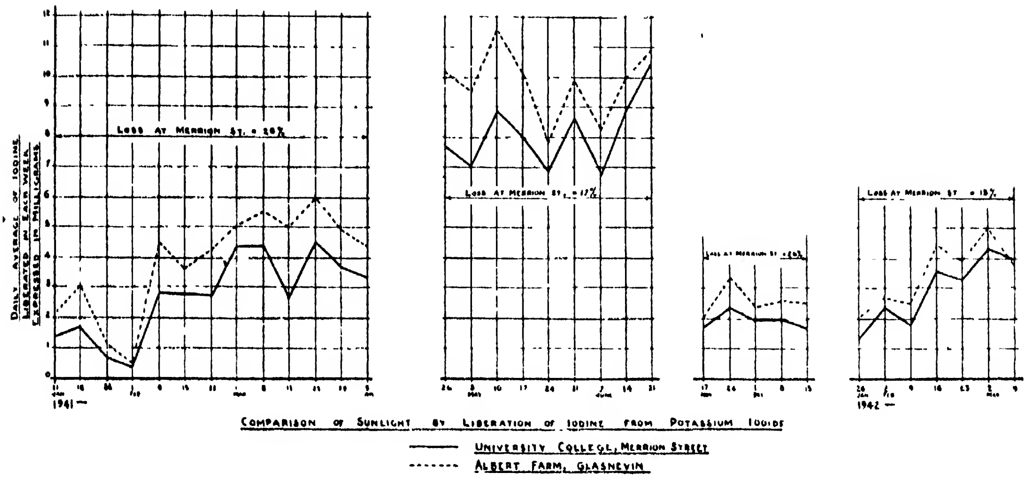

FIG. 5.

Legislation to Prevent Smoke Pollution.

Persons interested in the prevention of smoke pollution will find in the library of the Royal Dublin Society a copy of an ordinance which is now in force in the city of St. Louis, and which the corporation state has already solved for the most part the nuisance previously due to smoke in that city.

CHEMICAL DEPARTMENT,
UNIVERSITY COLLEGE,
DUBLIN

THE

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[SEPARATE ISSUE.]

No. 4. OCTOBER, 1942.

THE IDENTITY OF THE TOP-NECROSIS VIRUS IN UP-TO-DATE POTATO.

BY PHYLLIS E. M. CLINCH,
Albert Agricultural College, Glasnevin, Dublin.

(PLATE I.)

Price Two Shillings.

No. 4.

THE IDENTITY OF THE TOP-NECROSIS VIRUS IN UP-TO-DATE POTATO.

By PHYLLIS E. M. CLINCH,
Albert Agricultural College, Glasnevin, Dublin.

(PLATE I.)

[Read MAY 19. Published separately OCTOBER 6, 1942.]

TOP-NECROSIS was the term applied by Quanjer and Oortwijn Botjes (18) in 1929 to a specific type of potato virus disease characterised by necrosis of the inner phloem strands, death of the growing points, and eventual killing of the plant from the top downwards. The general term "streak" had previously been used in reference to all symptoms of a necrotic nature, irrespective of the location of the necrosis in the plant. It was recognised that more than one virus was capable of producing top-necrosis, and also that the range of varieties reacting in this manner differed for each of the viruses in question.

The presence of a top-necrosis ("streak") virus in apparently healthy Up-to-Date potatoes was detected in the course of intervarietal grafting experiments by Murphy and McKay (14) in 1926, a similar observation in regard to the American variety, Green Mountain, having been made by Schultz and Folsom (21) in the previous year. It is now known that practically all plants of these varieties are carriers of top-necrosis, and that the responsible virus is probably the same in both cases (16, 11). Other varieties are similarly infected, at least to some extent, e.g. Bliss Triumph, Burbank, Duke of York, and Earliest of All. So far as the writer is aware, no plant which is a carrier of this particular virus has been found free from virus X, as shown by the characteristic mosaic symptoms produced on inoculation to such hosts as *Datura Stramonium* and tobacco.

In 1931 Smith (24) recorded that analysis of the virus content of symptomless "streak"-carrying Up-to-Date yielded virus X and the insect-transmissible virus Y. Salaman and Bawden (20) reaffirmed this statement, but considered that the top-necrosis complex possibly contained a third virus (Z) in addition. However, the view that Up-to-Date "streak" is a complex of X and Y has not been confirmed by other investigators, nor has it been maintained at Cambridge. In 1933 Clinch and Loughnane (6) showed that the top-necrosis virus from Up-to-Date was filterable through an L5 Pasteur-Chamberland filter candle, and transmissible by sap inoculation to *Datura Stramonium* and tobacco. As no separation of viruses occurred in the course of these experiments, they suggested that the Up-to-Date contained only one virus, which was responsible for the top-necrosis in potato as well as for the mosaic symptoms in *Datura* and tobacco. Bawden (2) disagreed with this view

on the grounds that he could isolate from Up-to-Date a typical X virus which was non-necrotic to President and Arran Victory potato; he considered, therefore, that a second virus, probably distinct from X, must be responsible for the top-necrosis symptoms produced in potato. Bawden had no means of isolating the second virus, but designated it virus B (= *Solanum virus* 4 in Smith's (25) classification).

Dykstra (11, 12) stated that the top-necrosis virus could be separated from X in the Up-to-Date complex by grafting Up-to-Date on the U.S.D.A. Seedling 41956, which is immune to X but not to B. He recorded that virus B alone produced top-necrosis in President and Arran Victory, but caused no symptoms in tomato, *Datura Stramonium*, or pepper. He did not investigate any other properties of the virus. A fuller study of the properties of virus B (*Solanum virus* 4) was undertaken by Dennis (10), who also used Seedling 41956 for the isolation of the virus from Up-to-Date. Contrary to Dykstra's experience, Dennis found that the purified virus B which he recovered from Seedling 41956 produced conspicuous mosaic symptoms of the X type on many solanaceous hosts including tomato, *Datura Stramonium*, and pepper. The virus also reacted with anti-X rabbit serum, and its physical properties were identical with those of virus X. On the latter point, Dennis's results were somewhat at variance with those of Bawden (2), who had found that viruses B and X had similar thermal death points but differed in their resistance to "ageing" and dilution. According to Dennis the two viruses, B and X, differed only in their effects on certain potato varieties, and in the failure of either to protect against infection with the other. However, the ability of virus B to infect X immune Seedling 41956, if established, would obviously constitute a still more important point of distinction between the two.

The present writer has had occasion to examine the top necrosis virus from Up-to-Date in order to compare it with another top-necrosis virus related to F (8). In the course of this examination certain facts have come to light which appear to be of importance in establishing the identity of virus B, and the results are accordingly presented herewith.

Source of Virus and Symptoms in Other Hosts.

The stock of Up-to-Date potato used in this work was the same as that used by Clinch and Loughnane (6) in 1933, and has been grown continuously in an insect-proof glasshouse since that time. The plants are vigorous, and almost perfect virus carriers, displaying at times, however, a slight general mottling, especially when approaching maturity.

The reactions of other potato varieties when grafted with scions of Up-to-Date are as follows:—

(a) *Top-necrosis.*—Arran Cairn, Arran Chief, Arran Comrade, Arran Crest, Arran Pilot, Arran Scout, Arran Victory, Ballydoon, British Queen, Catriona, Doon Early. Dunbar Standard, Dunbar Yeoman, Early Regent;

Epicure, Gladstone, Golden Wonder, Katahdin, Kerr's Pink, King Edward, May Queen, President, Sharpe's Express, Ulster Monarch. (This group includes four varieties which react with top-necrosis to virus X, viz.: Arran Crest, Early Regent, Epicure, and King Edward.) (b) *Symptoms as in Up-to-Date.*—Arran Banner, Arran Consul, Arran Peak, Arran Signet, Bliss Triumph, Champion, Di Vernon, Duke of York, Dunbar Cavalier, Eclipse, Great Scot, Green Mountain, Majestic, Redskin. (c) *Crinkle.*—Irish Chieftain. (This variety is infected with virus A.)

Many of the foregoing varieties were tested at Cambridge as well as at Glasnevin, with similar results at both centres. Bawden (2) also records that the American varieties Burbank, Earliest of All, and American Wonder are carriers of the Up-to-Date virus.

Sap inoculation from Up-to-Date to other potato varieties.—It is generally stated that the Up-to-Date "streak" virus (= virus B) is not sap inoculable to potato, and is therefore less infectious than virus X. This, however, is only true of those varieties such as Arran Victory, British Queen, and President, which react to the "streak" virus with top-necrosis. Inoculation of these intolerant varieties is followed by the development of local necrotic lesions (Pl. I, Fig. 1), but the necrosis only rarely becomes systemic. The number of local lesions produced is greatly increased by sprinkling carborundum powder on the leaves before making the inoculation. Healthy plants of carrier varieties, i.e. those which react like Up-to-Date itself, can be infected with virus B just as easily as with virus X, and 100 per cent. infection of such varieties has been obtained in several different experiments.

On recording that inoculation of Arran Crest with Up-to-Date sap results in top-necrosis (which is also the effect of virus X in this variety), Clinch and Loughnane (6) suggested that the necrotic reaction could be due to the "streak" virus, i.e. virus B. Bawden (2), however, considered that the necrosis was due entirely to an X virus accompanying virus B in Up-to-Date, and stated that only virus X was recoverable from the necrotic foliage of the Arran Crest. That virus B may be introduced to Arran Crest by sap inoculation was shown in the following manner: Three healthy plants of the variety Arran Victory (necrotic to B, non-necrotic to X) were grafted with scions of healthy Arran Crest which, when fully established, were inoculated with Up-to-Date sap. Numerous local necrotic lesions developed all over the rubbed leaves. Later, the tops of the Arran Crest scions were cut, following which systemic necrosis developed in two of them. All three Arran Victory stocks developed "streak" symptoms, and *Datura* plants inoculated from them showed mosaic. Clearly, the "streak" virus entered the Arran Crest following inoculation, and passed eventually into the Arran Victory indicator stocks, although the experiment does not prove whether it or an accompanying X (or both together) is responsible for the top-necrosis in the Arran Crest.

Sap inoculation from Up-to-Date to other hosts.—The symptoms produced in *Datura Stramonium* and tobacco (*var.* white Burley) have been already

described by Clinch and Loughnane (6). They consist of a mosaic mottling identical with that caused by a mild strain of X (Pl. I, Fig. 2). The symptoms in tomato are also of the X type, and are distinctly mild; initial vein-clearing in the top leaves is followed by a slight dark and light green mottling, which later fades but is again visible on the lower leaves at maturity.

All plants of the above hosts which showed mosaic following inoculation were found to contain the top-necrosis virus when tested by grafting on suitable varieties of potato, such as President and Arran Victory.

Infection of a non-solanaceous host with virus B.—Having found that certain species of the common hemp-nettle, *viz. Lamium purpureum* and *L. hybridum* (Natural Order *Labiatae*), were susceptible to infection with virus X, tests were made to determine whether these species were also susceptible to virus B. Sap from "streak"-carrying Up-to-Date was inoculated to *Lamium hybridum* by the rubbing method, using carborundum powder. About three weeks later a mild mottle appeared in the leaves of the *Lamium* plants, and sub-inoculations from the mottled leaves to *Datura Stramonium* resulted in mosaic symptoms similar to those induced by direct inoculation from Up-to-Date. Scions from the *Datura* plants in turn produced top-necrosis when grafted on Arran Victory potato, showing that the virus content of the Up-to-Date was unchanged by passage through the non-solanaceous host. It has not so far been found that *Lamium* is susceptible to potato viruses other than X and B, but work in this direction is still in progress.

ATTEMPT TO ISOLATE THE TOP-NECROSIS VIRUS FROM UP-TO-DATE BY INFECTION OF U.S.D.A. SEEDLING 41956.

The stock of Seedling 41956 used in the tests was made available to this laboratory through the courtesy of Dr. T. P. Dykstra of Corvallis, Oregon. The variety was found to be highly susceptible to potato viruses Y, A, stipple streak, F, and a top-necrosis strain of F (8). Repeated attempts to infect it with either mild or severe strains of X were, however, entirely unsuccessful, a result which confirmed the statements of Schultz *et al.* (22), Dykstra (12), and Dennis (10) regarding the immunity of Seedling 41956 to virus X.

With a view to removing X from the supposed X + B complex in Up-to-Date, five plants of Seedling 41956 were grafted with Up-to-Date scions. After 34 days, when the scions had grown vigorously, each of the stock plants was tested for virus by grafting on President and Arran Victory potato, and by inoculation to *Datura Stramonium,* but with negative results. The tests were repeated after 84 days, but were again negative. Scions from the Seedling 41956 stocks were also grafted on President plants infected with mild X, on the assumption that B, if isolated in 41956, might require the addition of X in order to produce the top-necrosis reaction characteristic of the original Up-to-Date in President. In no case was a virus detected in the Seedling 41956 stocks, nor was any virus present in the tubers of these plants when they were

tested in the following year. The continued presence of the "streak" virus in the Up-to-Date scions after 84 days was, however, confirmed when the tops of the latter were grafted on healthy President, which reacted with top-necrosis. This proved that union with the tissues of Seedling 41956 had not affected the virus in the Up-to-Date scions.

In the following year three Seedling 41956 tubers were core-grafted from Up-to-Date, but the plants grown from them were virus-free. Four more 41956 plants were top-grafted as before, and 58 days later, when the Up-to-Date scions were 12–14 ins. high, shoots from each stock were grafted on President and Arran Victory. No symptoms developed in the latter, and when the tests were repeated after a further 18 days they were again negative. Four vigorous young 41956 plants were also inoculated with Up-to-Date sap, using carborundum powder. Four weeks later scions from each were grafted on healthy British Queen plants, but the latter failed to show any symptoms. All attempts therefore to infect Seedling 41956 with a virus from Up-to-Date were unsuccessful, although any other variety of potato could be infected with certainty by top-grafting.

It was understood from Dykstra's (11) preliminary statement on the subject that virus B was introduced without difficulty into Seedling 41956 by top-grafting with Up-to-Date, and was isolated in pure form, i.e. free from X, on Arran Victory potato and tomato by sub-grafts from the infected 41956. In a later paper (12) separation was said to have been effected by double-grafting Seedling 41956 and Up-to-Date on Arran Victory stocks, and this technique was accordingly tried by the writer. In the first experiment two healthy Arran Victory plants were grafted with Seedling 41956 scions which, when established, were further grafted with Up-to-Date scions. Both Arran Victory plants developed top-necrosis, which appeared after 10 and 18 days respectively. The intermediate 41956 scions were 2–3 ins long, and each had one foliage leaf. In a second experiment one Arran Victory and one British Queen were used as basal stocks, and the 41956 scions were allowed to become tall and extremely vigorous before being top-grafted with the Up-to-Date. The cutting of the tops at the time of grafting with Up-to-Date stimulated growth of the axillary buds on the 41956 stems, and these rapidly developed into strong leafy shoots. Eighteen days after grafting with Up-to-Date streak lesions appeared on the Arran Victory and British Queen stocks, and top-necrosis later ensued (Pl. I, Fig. 3). In further experiments the variety Arran Crest, which is intolerant of X, was used as the basal stock. Five Arran Crest plants were double-grafted, and the intermediate Seedling 41956 scions were in all cases 6–8 ins. long, with vigorous leafy shoots. Four of the Arran Crest plants developed top-necrosis, the streak lesions appearing in one case 13 days after grafting with Up-to-Date. The fifth Arran Crest failed to show symptoms, but in this case the Up-to-Date scion formed stolons at the base, and the axillary buds swelled up to form small aerial tubers, showing that downward translocation from the scion was inhibited.

The appearance of the top-necrosis in the basal stocks of Arran Victory

and British Queen confirmed Dykstra's (12) statement that virus B could pass through an intermediate scion of Seedling 41956 in a double graft It was found, however, that when sap from any of the basal stocks used (including Arran Crest) was inoculated to *Datura Stramonium*, mosaic symptoms developed which were identical with those produced by direct inoculation from Up-to-Date. The rate of infection from any one potato plant was comparatively low, and badly necrosed tissues which had dried out usually failed to produce symptoms. Altogether eleven out of thirty *Datura* plants inoculated from necrotic foliage showed mosaic, and scions from these plants produced top-necrosis when grafted on Arran Victory or British Queen. The symptomless *Datura* plants, on the other hand, were found to be virus-free. It appeared, therefore, that the Up to-Date virus complex had not been altered by passage through the intermediate scions of Seedling 41956. Tests were then made of the virus content of the 41956 intermediate scions Sap inoculation to *Datura* failed to produce symptoms, and subsequently scions from the 41956 branches were grafted on six Arran Victory plants. Although taken from double-grafted plants with flourishing Up-to-Date scions and severely necrotic basal stocks, these scions were all virus-free.

Further double-grafting experiments were carried out in which the Up-to-Date top scions were replaced by President scions infected with a severe strain of X, while Arran Victory and Arran Crest were used as the basal stocks. Notwithstanding the fact that in certain cases the intermediate 41956 scions were 12–14 ins. long, with vigorous leafy shoots, virus X in all cases passed from the top scions, and produced its characteristic symptoms in the basal stocks, but the 41956 did not itself become infected. These results show that Seedling 41956, inserted as an intermediate scion in a double graft, does not prevent the passage of virus X. The fact that the top-necrosis virus from Up-to-Date passed through Seedling 41956 in double grafts is therefore not proof that this virus is distinct from virus X.

EFFECT OF ANTI-X SERUM ON THE INFECTIVITY OF UP-TO-DATE SAP.

Recent work by Chester (5) and others has established the specificity of the reaction between plant viruses and their homologous antisera. It has also been shown that different strains of a given virus, however unlike symptomatically, are serologically indistinguishable at least with the ordinary precipitin technique. Spooner and Bawden (26) found no antigenic differences between different strains of potato virus X, and demonstrated incidentally that sap from Up-to-Date potato carrying a mild strain of X flocculated with anti-sera to the S and G strains of X derived from tobacco No flocculation occurred when anti-X sera were mixed with saps containing viruses unrelated to X. It might be expected, therefore, that if Up-to-Date sap contains two distinct viruses, X and B, the X could be removed from the mixture by neutralization with its specific immune serum. This technique was successfully employed by Cohen (9)

to purify tobacco necrosis virus from admixture with tobacco mosaic virus. Cohen also found that the removal of the tobacco mosaic virus from the mixture by this method entailed no apparent loss in the amount of tobacco necrosis virus.

The anti-X serum used in the present work was obtained from rabbits which had received a series of 6 intravenous injections with a mild strain of virus X from President potato. Preliminary precipitin tests with healthy and X-infected sap of British Queen potato showed that the serum reacted well with the X sap, but gave no precipitate with the healthy juice. A series of tubes was prepared, each containing 2 c.c. of antiserum at dilutions (in 0 85% saline) of 1 in 2, 1 in 4, 1 in 8, and 1 in 16, respectively, control tubes receiving saline only. The tubes were well shaken, incubated at 37° C. for 1½ hours, and then placed in the refrigerator for 1 hour. Only those containing anti-X serum showed flocculation. The tubes were then centrifuged for 10 minutes, and the supernatant fluids inoculated to *Datura Stramonium*. The mixtures containing serum dilutions 1 in 2, 1 in 4, and 1 in 8 failed to produce symptoms in *Datura*, showing that the X virus had been completely neutralized. Scions from these symptomless *Datura* plants were then grafted on healthy Arran Victory potato plants, but these failed to show any reaction, indicating that the virus responsible for top-necrosis had also been removed.

The antigen solutions used in these tests were not highly purified like those of Cohen, and the possibility must be considered that even if virus B were a separate virus, serologically distinct from X, it might be carried down mechanically in the serum-virus X precipitate. It seems unlikely, however, that a resistant virus existing in fairly high concentration would be completely removed in this manner, and the experiment strongly suggests that the top-necrosis virus is actually neutralized by the anti-X serum.

Physical Properties of the Up-to-Date Top-Necrosis Virus.

On account of the failure to isolate virus B from the supposed X + B complex in Up-to-Date, tests of the physical properties of the virus were made with crude Up-to-Date sap. The juice was subjected to the desired treatments, and each treated sample (and untreated control) inoculated to 4–6 *Datura* plants. Scions from the latter were subsequently grafted on healthy Arran Victory plants, whereby the presence of virus B could be detected.

Filterability.—Virus B passed freely through L 1, L 3, and L 5 grades of Pasteur-Chamberland filter candles.

Thermal inactivation point.—68°–70° C. (10 mins. exposure).

Longevity in vitro.—Varied with different samples taken at different times. The crude sap was stored in stoppered bottles in the dark at 17°–20° C. In some cases infectivity was lost after 28 days, while in others infection was secured after 35 but not after 45 days. The approximate time for complete inactivation under the conditions mentioned is about 30 days.

Resistance to dilution.—50% infection was obtained at a dilution of 1 in 10,000, none at 1 in 100,000.

In general, these properties are essentially similar to those recorded for viruses of the X type. Furthermore, in the course of these tests virus B was recovered from all *Datura* plants which showed mosaic symptoms following inoculation with treated sap, while symptomless *Datura* plants were found to be virus-free when grafted on Arran Victory. Consequently, if two viruses are present in the Up-to-Date sap, one responsible for the top-necrosis and the other responsible for the mosaic symptoms in *Datura*, both must have identical physical properties. Actually the results fail to indicate that more than one virus is present, and are not in agreement with Bawden's statement (2) that virus B is destroyed and that virus X alone is recovered from Up-to-Date sap after 4 weeks' "ageing" or at a dilution of 1 in 1000.

Immunological Relationship of Viruses X and B.

In order to test whether virus X exercised protection against virus B, sap from Up-to-Date was inoculated to X-infected potato plants of three different varieties. These included Majestic, which is a carrier of virus B, and President and British Queen, both of which react to B with necrotic symptoms. The plants used in these experiments were young, growing actively, and of uniform size. Except where otherwise mentioned carborundum powder was sprinkled on the leaves before rubbing them with the infective sap.

(1) Six healthy plants of Majestic and six infected with a mild strain of X were inoculated with Up-to-Date sap on 24th March. After about 14 days the healthy plants developed pale spots on the upper leaves, and later a diffuse mild mottle similar to that in the X-infected plants. The latter showed no change due to the inoculation. After 38 days a scion from each plant was grafted on Arran Victory; the six previously healthy plants caused top-necrosis, but the X-infected plants produced only a mild mosaic mottle. The X-infected plants, therefore, had resisted infection with the "streak" virus, while the healthy plants were fully susceptible.

(2) Six healthy President and six infected with a mild X strain were inoculated as above. The two lots were grown on either side of a glasshouse compartment, which was partially shaded so as to minimise hardening of the foliage. After about 14 days small local lesions appeared on the healthy plants, and these gradually increased in size to a diameter of 5–7 mm., and then dried out. In the meantime a second inoculation was made to the next three leaves above those already inoculated, and these in turn showed similar lesions. Not a single lesion appeared on any of the X-infected plants under the same conditions. Twenty-eight days after inoculation the upper leaves of the originally healthy plants were tested by inoculation to *Datura Stramonium*. Two of the six plants were found to contain a mild X virus which was non-necrotic to Arran Victory. Later the mottle was visible in the potato plants

themselves. This experiment was repeated exactly with twelve more President plants. Again, the six X-infected plants failed to show local lesions, while all the healthy plants did so. In addition one of the healthy plants later became systemically infected with a mild X virus.

(3) Three healthy British Queen plants and three infected with virus X were inoculated with Up-to-Date sap on 23rd May. The local necrotic reaction of British Queen to Up-to-Date sap is much more pronounced than that of President, possibly because the British Queen foliage is much more sappy. Five days after inoculation numerous minute necrotic lesions appeared on the three healthy plants, and these rapidly developed into distinct greyish lesions, which coalesced and eventually caused the leaves to turn yellow and die; not a single lesion appeared on the X-infected plants. Nineteen days after inoculation the upper leaves of the healthy plants were tested on *Datura*, but no symptoms were produced. After a further 10 days one of the plants showed systemic streak lesions in the lowest axillary shoots, then in the next shoots above, and so on until lesions were visible in the top leaves. These lesions tended to dry out, they were accompanied by pale blotches, and eventually the foliage presented a strongly mottled appearance with accompanying necrotic lesions. The growing points were not killed, and the advance of the disease was distinctly acropetal and associated with the development of axillary shoots. The other two plants also showed slight systemic streak symptoms, which did not appear for more than 6 weeks after inoculation and were confined to a few isolated necrotic lesions associated with the veins of the leaves.

In a further experiment six healthy British Queen plants and six infected with X were inoculated with sap from "streak"-carrying Up-to-Date. In addition six healthy British Queen plants were inoculated with a mixture of sap from "streak" carrying Up-to-Date and from Up-to-Date free from virus B but carrying a mild X. Four leaves on each of the eighteen plants were smeared with inoculum, but only two leaves in each case were sprinkled with carborundum powder. All plants were grown under the same conditions. Four days after inoculation numerous minute necrotic local lesions appeared on the leaves of the twelve healthy plants to which carborundum powder had been added (Fig. 1), and these rapidly increased in size, coalesced and dried out. Very few lesions developed on the leaves which had not received carborundum powder. As before, the leaves of the X-infected plants failed to show a single lesion. Twenty-one days after inoculation, an upper leaf from each of the twelve previously healthy plants was removed and the sap inoculated to two *Datura Stramonium* plants. The results showed that only one out of the six British Queen plants inoculated with "streak" Up-to-Date sap was systemically infected with mild X virus, while all six plants inoculated with the mixture of Up-to-Date saps were so infected. Later, following cutting back, top-necrosis developed in one of the plants inoculated with "streak" sap only, while mosaic developed in another.

The results of the foregoing experiments show that of twenty-one X-infected potato plants inoculated under optimum conditions with sap from Up-to-Date, not a single one showed a trace of infection with virus B, although healthy control plants inoculated at the same time all showed evidence of infection with the necrotic virus. The systemic development of a non-necrotic X virus in some of the control plants, which will be discussed later, does not alter the fact that virus B cannot be introduced by sap inoculation to potato plants already systemically infected with virus X; in other words virus X protects against virus B, and the two must be regarded as related strains. In a single immunity experiment in which *Datura Stramonium* was used the results confirmed those obtained with potato. Four out of a batch of eight *Datura* plants were inoculated with virus X, and the remaining four, which were to serve as controls, were inoculated with virus F, which is unrelated to X. Fifteen days later all the plants were inoculated with sap from streak-carrying Up-to-Date, and after a further 26 days tested by grafting on healthy Arran Victory potato. Only the four control *Datura* plants had become infected with virus B, and caused top-necrosis in Arran Victory.

The immunity conferred by virus X against virus B does not hold if the second virus be introduced by graft. While there is a distinct resistance to the entry of the second virus, it seems that this resistance is usually overcome when a constant supply of virus from a grafted scion is maintained. The top-necrosis, however, takes considerably longer to develop in X-infected plants than in healthy plants grafted under the same conditions, and sometimes the necrosis is restricted to the tips of the axillary shoots and fails to kill the growing points or to affect the foliage generally. Similarly, when scions infected with a severe strain of X were grafted on potato plants already infected with mild X, the severe strain had obvious difficulty in becoming established in the stock plants, but eventually did so and also penetrated the tubers. Bawden (1) previously reported that virus D, now known to be a strain of X, could be introduced by grafting but not by sap inoculation to potato plants already infected with virus X.

Bawden (2) and Dennis (10) both reported that virus X failed to confer immunity against the Up-to-Date streak virus. Part of the evidence for this statement was derived from experiments carried out with potato plants in which the second virus was introduced by graft. For reasons given in the preceding paragraph it is clear that the results of such experiments are unreliable. In the case of a single potato plant which was sap-inoculated with the second virus Dennis (10) found that previous infection with his *Solanum virus* 4 did not protect against further infection with virus D (1). In experiments with *Datura Stramonium*, Bawden (2) found that 11 out of 18 X-infected plants became infected with B when reinoculated with "streak" Up-to-Date sap, and concluded that the two viruses were not mutually protective. Dennis (10) supported Bawden's conclusion, stating that virus X in *Datura* did not exert any protective action against the *S. virus* 4 isolated by him from

Seedling 41956. Nevertheless, it would appear from Dennis's description that the full severe symptoms of his *S. virus* 4 were not produced when that virus was inoculated to *Datura* plants already infected with one or other strain of X, although a certain degree of alteration in the symptom picture resulted from the second inoculation. Furthermore, when tobacco plants infected with *S. virus* 4 were reinoculated with the intensely necrotic N strain of virus X (19), the full symptoms of the latter seem to have occurred in only one out of 10 plants inoculated. Unless *S. virus* 4 is unique among potato viruses in that it is less severe in combination than alone, these results seem to suggest that some measure of protection was afforded by the preliminary virus in the majority of these plants.

Dennis states that in his experiments the second virus was introduced 9 days after the first, a period which, in the writer's opinion, may have been insufficient to allow of complete invasion of the plants by the "protecting" virus. In two different tests carried out with young actively growing *Datura* plants it has in fact been found that, although vein-clearing was visible in the top leaves of all plants within 6–7 days after inoculation with a mild strain of X, complete invasion had not yet taken place in the majority of the plants after 9 days; the larger leaves, or parts thereof, still reacted with necrotic lesions to a severe strain of X. While it is possible that the initial virus might become completely systemic in young seedlings in less than 9 days, it appears safer to use potato plants grown from infected tubers for immunity experiments of this type; or else to extend the period between the first and second inoculations.

Isolation of a Non-Necrotic Virus X from "Streak"-Carrying Up-to-Date Potato.

It has been shown above that out of 21 healthy potato plants of the intolerant varieties President and British Queen which were inoculated with "streak"-carrying Up-to-Date sap and which showed local necrotic lesions, only 3 British Queen plants developed systemic streak symptoms. Five of the remaining 18 plants, however, eventually developed systemic infection with a mild, non-necrotic X virus. Bawden (2) had previously reported that President plants inoculated with Up-to-Date sap usually developed systemic infection with virus X, and also (as already mentioned) that a similar non-necrotic X could be isolated from Up-to-Date by certain physical treatments of the sap. He accordingly assumed that two distinct viruses are present in Up-to-Date, *viz.* virus X, which becomes systemic in President, and virus B, which remains localized in the lesions on the inoculated leaves.

In the present work the development of a non-necrotic X virus in the tops of potato plants inoculated with Up-to-Date sap was the exception rather than the rule. If such a virus co-exists with the top-necrosis virus in Up-to-Date, one would have expected a higher rate of infection, seeing that X is easily transmissible by sap inoculation to President and British Queen. The idea that virus X in the inoculum might in some cases be destroyed in the local lesions

caused by virus B was rejected in view of the high rate of infection obtained when sap containing mild X was mixed with the "streak" Up-to-Date sap before inoculation. Under the circumstances, and in view of the strong evidence that virus B is itself a form of X, the possibility must be considered that the non-necrotic form develops in the inoculated plants as a variant of the necrotic one. Price (17) found that new strains of cucumber mosaic could arise in local lesions on cowpea leaves, as well as in yellow spots on systemically diseased leaves. Salaman (19) also records that virus X was converted from a severe to a mild form in local lesions on certain non-solanaceous plants. It is true that such changes do not usually occur with any degree of regularity, and while the simpler explanation that two virus strains exist in Up-to-Date may be the correct one, nevertheless the possibility of strain variation in the local lesions cannot be excluded.

MODE OF TRANSMISSION OF THE TOP-NECROSIS VIRUS FROM UP-TO-DATE.

In a previous paper (6) it was reported that attempts to transmit a virus from Up-to-Date using *Myzus persicae* as vector were unsuccessful. The host plants used were President potato and *Datura Stramonium*. No further insect transmission experiments have been carried out, nor has there been any record of successful insect transmission by other workers, so far as the writer is aware.

In an examination of the spread of viruses into healthy potato stocks in the field, Clinch, Loughnane, and Murphy (7) found 4% infection with virus B in one crop of Champion and two crops of Arran Banner: the infected plants also gave mosaic symptoms typical of X in *Datura Stramonium*. Loughnane and Murphy (13) recorded accidental infection of a potato plant (*var.* Monocraat) with virus B. As the plant was grown in an insect-proof glasshouse, they presumed that infection took place as a result of contact with a B-infected plant. The present writer had occasion to grow Irish Chieftain potato plants alongside Up-to-Date plants in the glasshouse. In the following season the tubers of one Irish Chieftain gave rise to plants infected with crinkle (15), indicating the addition of X to the A already present in Irish Chieftain. When grafted on President, scions from the crinkle Irish Chieftain produced top-necrosis. There can be little doubt that this was another instance of transmission of B through leaf contact.

While definite experiments on the transmission of B by leaf contact have yet to be carried out, the evidence so far available suggests that B is probably transmitted under natural conditions in the same way as X, *viz.* by contact between leaves of adjacent healthy and diseased plants.

DISCUSSION AND CONCLUSIONS.

The results of the present investigation appear to furnish convincing evidence that the top-necrosis virus in Up-to-Date ("streak" virus, virus B,

Solanum virus 4) is a strain of virus X. It is similar to X in transmissibility, host range, physical properties, and in its inability to infect X-immune Seedling 41956; there is also some evidence that it is indistinguishable from X serologically. Furthermore, it cannot be introduced by sap inoculation to potato plants already infected with virus X. It differs from typical virus X only in that it causes top-necrosis in a number of potato varieties not so affected by the typical strain, a difference which recently has been shown to exist between two strains of potato virus F (8). It is possible that a non-necrotic strain of X may co-exist with the top-necrosis virus in Up-to-Date, but, as pointed out in the text, the evidence in support of this conclusion is capable of another interpretation; in any case the co-existence of two or more strains of X in potato plants is apparently quite common in Nature (19).

The conclusions reached are not in accordance with those of other investigators, who took the view that Up-to-Date contains two distinct viruses, virus X responsible for the mosaic symptoms in *Datura Stramonium*, tobacco, etc., and the top-necrosis virus which Bawden (2) designated B. Bawden found that the thermal inactivation point of the top-necrosis virus was identical with that of X, and this property has, up to the present, proved to be a very reliable index of relationship between viruses Bawden's main reasons for concluding that the top-necrosis virus was distinct from X were (*a*) because he could isolate a non-necrotic X from Up-to-Date, and (*b*) because X failed to confer immunity against B. The isolation of a non-necrotic X is not proof that the top-necrosis virus is not also a form of X, and his evidence therefore rests entirely on the absence of cross-immunity between the two viruses, which has not been confirmed in the present investigation.

The strongest reason for regarding B as a virus distinct from X was advanced by Dykstra (11) when he stated that B could infect Seedling 41956, which has been repeatedly shown to be immune to X. In the present work it was found impossible to infect 41956 with any virus from Up-to-Date, either by top-grafting, core-grafting, or sap inoculation. It would be possible to explain the discrepancy between the two sets of results if it could be assumed that all Dykstra's infections of 41956 were carried out by the double-grafting method, as described in his second report (12). It has been shown that when Up-to-Date is double-grafted on 41956 and a susceptible basal stock, the virus (or viruses) present in the Up-to-Date passes unchanged through the 41956, but does not infect it; the same holds true when President infected with virus X is used as an upper scion. In other words, no resistance is offered to the passage of virus X or the Up-to-Date virus by X-immune Seedling 41956 when the latter is inserted as an intermediate scion between two susceptible varieties. To prove the absence of X in his Arran Victory basal stocks, Dykstra inoculated from the necrotic foliage to *Datura Stramonium* and pepper, which showed no symptoms. However, Smith (23) recorded in 1928 that inoculation from necrotic shoots of Arran Victory potato which had been grafted with Up-to-Date failed to produce symptoms in tomato plants, and it is the writer's experience that

sap inoculations from plants showing top-necrosis may be successful, but more frequently yield negative results, even though the responsible virus may be one which is easily sap-transmissible. For this reason one might suggest that Dykstra reached an erroneous conclusion regarding the virus content of his basal stocks by relying on the negative results of tests with necrotic foliage. It must be pointed out, however, that in his preliminary report (11) Dykstra specifically states that virus B was conveyed to 41956 by top-graft, and then to tomato by sub-graft from 41956.

Apart from the identification of B as a strain of X, the behaviour of Seedling 41956 in allowing the passage of virus X through its tissues in the double-grafts, without itself becoming infected, is interesting. Clearly, virus X is unable to multiply in 41956, although other potato viruses can do so. The reason for this is unknown, but assuming that the virus is a chemical substance of protein nature (3), it may be that some ingredient or physical condition essential for its synthesis is lacking in the cells of this variety. It appears unlikely that the immunity of 41956 to virus X is due to destruction of the virus by antibodies or other toxins present in the tissues. If such substances are present at all (and it has been found impossible to demonstrate them in vitro) they cannot be operative to any extent in the stem in view of the fact that virus X can survive passage through 12–14 ins. of immune Seedling stem in the double-grafted plants. If virus X does not multiply in Seedling 41956, it follows that its movement through the tissues of this variety is not the result of an autocatylitic reaction; it seems probable that the particles of virus X are merely carried passively downwards from the upper scion through the Seedling 41956 into the susceptible basal stocks, where they multiply freely. The same applies to the top-necrosis virus from Up-to-Date. There is no reason for not believing that this is also the mode of transport of these viruses in the stems of susceptible varieties, and it is noteworthy that the time taken for the development of symptoms in the basal stocks of double-grafted plants was of the same order as in single-grafted plants. Furthermore, it has been pointed out in the present paper that where food translocation from an Up-to-Date scion appeared to be impeded, no virus passed into the basal Arran Crest stock, although the scion remained alive. Clinch and Loughnane (6) previously noted that when a small tuber formed at the base of an Up-to-Date scion grafted on healthy President potato, no disease appeared in the stock plants during a period of seven weeks following grafting. Removal of the tuber, however, with the consequent diversion of the food stream from the tuber reservoir into the President stem, resulted in the development of top-necrosis 10 days later. This indicates that the downward passage of the Up-to-Date virus in grafted plants is directly connected with the movement of elaborated sap. In this connection a recent review by Bennett (4) of the evidence indicating a correlation between virus movement and food translocation is of considerable interest.

Dennis (10), as well as Dykstra, claimed to have infected 41956 with the top-necrosis virus from Up-to-Date (*Solanum virum* 4), and by this means to

have freed it from the X virus believed to accompany it. As already mentioned, the virus thus isolated by Dennis produced extremely severe symptoms of the X type in *Datura Stramonium*, tomato, and several other solanaceous hosts. In physical properties, as well as serologically, it was found to be identical with X; the two viruses, however, were not mutually protective. Dennis's results are therefore completely at variance with those of the present author who has so far failed to infect X-immune Seedling 41956 with any virus from Up-to-Date, or with any virus resembling X.

The author is indebted to Dr. R. McKay, University Lecturer in Plant Pathology, and Mr. J. B. Loughnane, with whom various points in connection with this work have been discussed. Grateful acknowledgment is also made to Professor W. J. Kearney, M.R.C.V.S., for his valuable help in performing the rabbit injection experiments.

Summary.

Of 39 varieties of potato which have been tested, the majority react with top-necrosis when grafted with scions from "streak"-carrying Up-to-Date potato. The remainder show a transient mottle similar to that in Up-to-Date itself, excepting Irish Chieftain, which carries virus A, and which reacts with crinkle (X + A).

Sap inoculation of the "streak" virus (syns. virus B, *Solanum virus* 4) from Up-to-Date to healthy potato plants of intolerant varieties results in local necrotic lesions, but the necrosis rarely becomes systemic. The virus is easily transmitted to potato plants of tolerant varieties.

Sap inoculation from Up-to-Date to *Datura Stramonium*, tobacco and tomato, as well as to *Lamium hybridum* (Order *Labiatae*), results in mild mosaic symptoms of the type characteristic of virus X; the presence of the "streak" virus in the infected plants was demonstrated by return grafts to potato.

All attempts to infect X-immune Seedling 41956 with a virus from Up-to-Date, or with virus X, were unsuccessful, but the variety was readily infected with potato viruses unrelated to X.

When Seedling 41956 was inserted as an intermediate scion between Up-to-Date and a susceptible basal stock in double-grafted plants, the virus content of Up-to-Date passed unchanged into the basal stocks. In similar double grafts in which the Up-to-Date top scion was replaced by X-infected President, no resistance was offered by the intermediate 41956 scion to the downward passage of X. The 41956 scions themselves failed to become infected with X, or with any virus from Up-to-Date.

Removal of X from Up-to-Date sap by precipitation with anti-X rabbit serum resulted in a simultaneous removal of the virus responsible for "streak."

The physical properties of the Up-to-Date "streak" virus are similar to those recorded for viruses of the X type.

The "streak" virus could not be introduced by sap inoculation to potato or *Datura Stramonium* plants already infected with virus X. The immunity of X-infected plants to the "streak" virus or to other strains of X does not hold if the second virus be introduced by graft.

Following inoculation with "streak" Up-to-Date sap, healthy potato plants of intolerant varieties occasionally developed systemic infection with a non-necrotic X virus, whereas similar plants inoculated with a mixture of "streak" sap and X-infected sap all developed systemic X infection. The possibility that in the former case the non-necrotic X may develop as a variant of the Up-to-Date "streak" virus in the local lesions is suggested.

The conclusion has been reached that the "streak" virus (virus B, *Solanum virus* 4) in Up-to-Date potato is a strain of virus X, and differs from typical X only in its effects on certain varieties of potato. Whether or not a non-necrotic strain of X co-exists with the top-necrosis strain in Up-to-Date is not certain.

The nature of the immunity of Seedling 41956 to virus X, and the probable mode of transport of X in potato stems is discussed in the light of the present results. A correlation between movement of the Up-to-Date virus and food translocation is indicated.

EXPLANATION OF PLATE I.

FIG. 1.—Leaf of healthy British Queen plant showing local lesions as a result of inoculation with sap from "streak"-carrying Up-to-Date potato.

FIG. 2.—Leaf of *Datura Stramonium* plant, showing mosaic symptoms following inoculation with Up-to-Date sap.

FIG. 3.—Double-grafted potato plant showing A upper scion of Up-to-Date, B intermediate scion of X-immune Seedling 41956, and C basal stock of British Queen. Note large, vigorous branch of 41956 scion, and top-necrosis in several shoots of basal British Queen stock.

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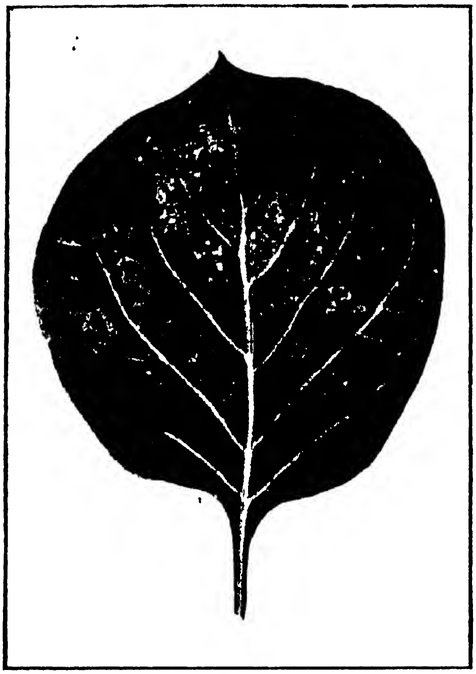


FIG. 1.

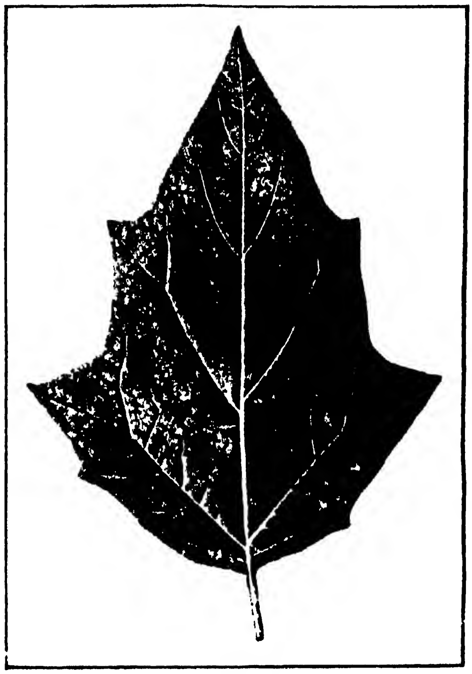
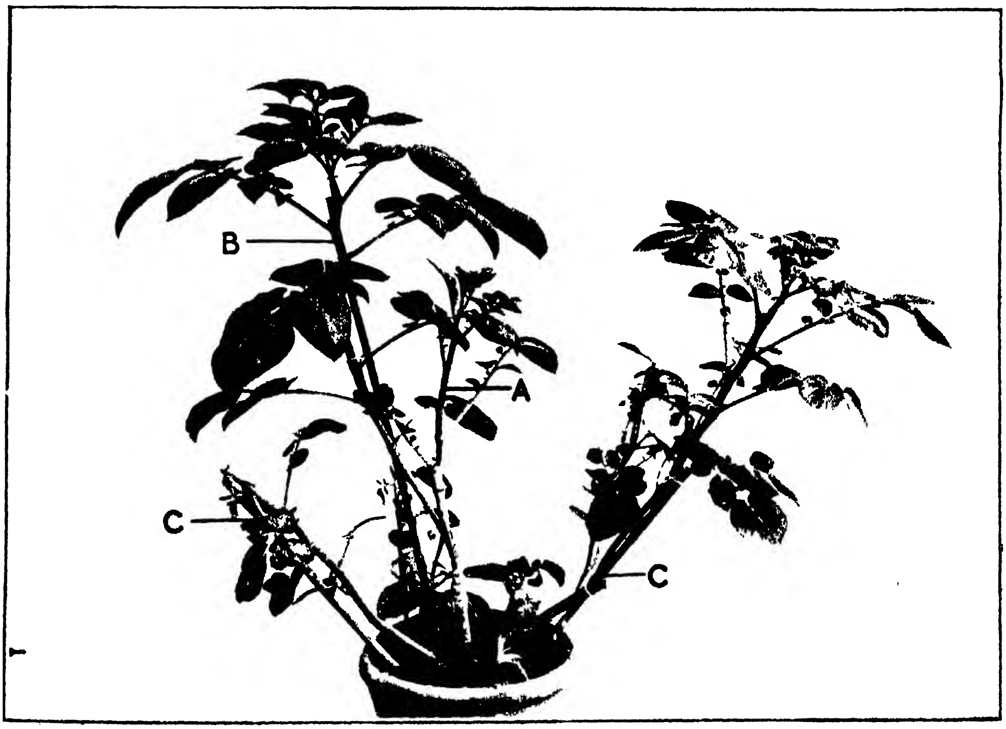


FIG. 2.



Photographs FIG. 3. *G. H. McLean*

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POLLINATION IN *TSUGA PATTONIANA* AND IN SPECIES OF *ABIES* AND *PICEA*.

By JOSEPH DOYLE and ANN KANE,

University College, Dublin.

(Plates 2 and 3.)

Price Three Shillings.

No. 7.

POLLINATION IN *TSUGA PATTONIANA* AND IN SPECIES OF *ABIES* AND *PICEA*.

By JOSEPH DOYLE and ANN KANE,
University College, Dublin.

(Plates 2 and 3.)

[Read November 24, 1942. Published separately March 15, 1943.]

Observations were published some years ago (Doyle and O'Leary, 1935 a, b) on pollen reception, and micropyle structure in relation to it, in certain members of the Pinaceae (then referred to as the Abietineae). These observations were made on certain representative species of the genera *Cedrus, Larix, Pinus, Pseudotsuga,* and *Tsuga.* The present paper supplements these observations, with some along the same lines on certain species of *Abies* and *Picea* and on an additional important species of *Tsuga*; and extends still further the range of types of pollination mechanism within the family. To avoid nomenclature confusion the specific names here used are those in Pilger (1926), without, however, implying any opinion as to their correctness.

TSUGA PATTONIANA.

Doyle and O'Leary (1935 b) have already described the pollination phenomena in *Tsuga heterophylla* and *Ts. dumosa* (= *Brunoniana*). In both pollen falls on the cone scales, the tubes growing along these to reach the micropyles. These two species are included by Pilger (1926) in the section *Eutsuga,* and probably all the species belonging to this section would show the same behaviour, as all seem to share the character of wingless pollen grains. There is, however, at least one other true species in which the grains *are* winged, viz., *Tsuga Pattoniana.* It differs also in certain structural features from most of the members of the genus, and is frequently separated in the section *Hesperopeuce.* It was obviously of interest to determine the pollen behaviour in this case.

It is still uncertain whether *Tsuga Pattoniana* is the only species with winged grains. Thus there have recently been described certain new species of *Tsuga* which, being amphistomatic, may properly belong to the section

Hesperopeuce. These species are *Ts. longibracteata* and *Ts. crassifolia,* the latter possibly identical with *Ts. Pattoniana.* Details of these and other species can be found in the recent revision of the genus by Flous (1936). No data, however, are anywhere given of the nature of the pollen grains; and no fertile specimens, living or herbarium, are available here. There is also *Ts. Jeffreyi* Henry. This species was for a long time described as a variety of *Ts. Pattoniana,* but is now commonly given specific rank, and is considered to be a fertile hybrid—*Ts. Pattoniana* × *heterophylla.* Some living specimens are growing in English gardens, but in this case also it proved impossible to locate or obtain fertile material for personal examination nor could any record be found of the nature of its pollen grains. A cross between species, one (*heterophylla*) with winged grains, and one (*Pattoniana*) without wings, would repay examination, especially in view of the fact that in *Ts. Pattoniana* the structure of the ovule and the pollen behaviour are different from those of *Ts. heterophylla,* as the description below brings out. It is hoped to examine this point if occasion offers.

It is of further interest in this connection that Wodehouse (1935) in his book on pollen grains seems to have overlooked the existence of *Ts. Pattoniana,* and considers wingless pollen grains characteristic of the whole genus. "Apparently," he says (p. 267), "there is in existence to-day no intermediate form between that of the wingless *Tsuga* grain and the winged form of the Abietineae." He refers, however, to fossil species of *Tsuga* from the Tertiary which bear rudimentary bladders, and concludes, probably quite justly, that the present form of the wingless *Tsuga* grain is derived from an ancestral winged type.

Specimens of *Ts. Pattoniana,* though not common, are to be found in many gardens in Ireland and Britain, but cones are rarely produced. No coning tree could be traced in Ireland during the period in which the observations recorded in this paper were being made, and unsuccessful application was made to many gardens in Britain where specimens were known to exist. We greatly appreciate the fact that, at all the places written to, the gardeners or owners made diligent search for the young cones, which are small and inconspicuous. Happily, however, a tree in Westonbirt carried a few cones in 1936 and in 1939. Our best thanks are due to Mr. W. J. Mitchell, the head gardener, for his trouble in locating, securing, and forwarding, in excellent condition, a number of cones at suitable intervals. These cones, though relatively few in number, were sufficient for our purpose.

Pollination takes place in *Ts. Pattoniana* early in the third week in May. The young ovules, at this time, are shown in adaxial view in Pl. **2,** 1, and in abaxial view in Pl. **2,** 2. The micropyle area differs markedly from that of the two species already described (Doyle and O'Leary, 1935 b, Pl. **4,** 1). The integument here shows a lobed flaring expansion adaxially, while on the abaxial side a broad slit runs down a short way to expose the tip of the nucellus. The shape, degree of lobing, and extent of spread of the flare, vary, as is shown

in the two ovules in Pl. **2**, 1. The ovule on the left shows a commoner type, but the flap may be more splayed in some cases. This flap is composed of typical succulent and semi-translucent stigmatic tissue, and is undoubtedly a stigmatic expansion which partially blocks the passages between the cone scales. When pollination is effected the grains are found adhering to various parts of the stigmatic area—on the edge, well in towards the nucellus, and also on the outer surface. In spite of the shape of the ovule, grains are rarely found actually on the nucellus. Comparatively few remain lodged on the scales, in marked contrast to the behaviour in the other species.

The condition about five weeks later is shown in Pl. **2**, 3. The dissected ovule on the right, in which the archegonia are faintly indicated, shows that development has proceeded rapidly. Actually, in the collection of this date, stages were found from male cells just before fertilization to two-tiered proembryos. Little change has taken place in the stigmatic area, except that it has become flattened on the shoulders of the ovule as this presses, in growth, against the axis and the neighbouring scales; or part of it may, in some cases, be turned over across the micropyle. Shortly after pollination growth of the nucellus pushed its tip a little higher, so that at the stage here shown, particularly in the dissected ovule on the right, it fits into the actual micropyle opening like a plug. Its upper cells take on something of the appearance of a stigmatic tissue in preparation for the reception of pollen tubes. A receptive zone in the upper part of the nucellus is fairly common, even in species in which the pollen lodges directly on it, but the development here is more distinct and comparable to that in the araucarians; the nucellar flare in *Saxegothaea* is an extreme expression of this. The figure also shows that the pollen has germinated where it fell, the tubes growing, fairly directly, to reach and pierce this receptive area. Lodgment on the stigmatic surface of the micropylar expansion is, however, not essential for pollen germination. In the left of the figure a couple of grains which lodged near, but not on, the stigmatic area are shown forming tubes which successfully reached the nucellus. In the case of grains further away, the one on the extreme left for example, although germination may take place, the growth of the pollen tube is not so definite in direction. The general tendency of growth is along the scale, or the surface of the ovule, towards the cone axis, but no tubes have ever been seen to reach the nucellus.

Tsuga Pattoniana thus shows still another type of pollination mechanism in the Pinaceae. It differs from that described for other species of *Tsuga* mainly in (*a*) the presence of wings on the grain, (*b*) the development of a stigmatic flare round the micropyle, and (*c*) the poorer growth power of the pollen tubes.

ABIES.

Pollination phenomena in this genus have been referred to by Strasburger (1871) for the common fir and for *A. balsamea.* He says that ovular structure

in *Abies* closely resembles that in *Larix*. The edge of the micropyle is said to be expanded into a one-sided helmet-shaped outgrowth, which only differs from that in *Larix* "durch einen, oft schwachen, mittleren Einschnitt." The pollen grains "werden in das Innere eingesogen," and come to lie in a depression on top of the nucellus. Exactly how this happens is not described, nor is any specific mention made of a fluid secretion. He further states that the edges of the integument do not later dry up or curl inwards, but can be seen for a long time unchanged in form and position, the micropyle remaining open. This account, while in a way correct, is on the whole misleading and inaccurate, and is probably the result of too rapid and cursory observations. Goebel (1933) also mentions *Abies*, but very briefly. He refers micropylar form to three classes—the tubular, the pincers, and the stigmatic types. The tubular type is the common one, as seen in the Cupressaceae, Taxaceae, etc., and is associated with the secretion of the well known pollination drop. The stigmatic type is that of *Larix* and *Pseudotsuga*. The pincers type, he says, is probably characteristic of most Abietineae (Pinaceae as used in this paper), and is shown by *Pinus* and *Abies*. This statement is also incorrect, as *Abies* in structure and behaviour is quite different from *Pinus*.

Several species of *Abies* were observed, and all showed the same general features. Certain species showed minor differences, but as *A. Nordmanniana*, being more accessible, was more closely observed than the others, its description is taken as a type, only brief reference being made to some of the others.

(A.) *ABIES NORDMANNIANA.* Pollen is shed on the average between the 5th and 12th of May. The ovulate cones are already fairly large, 5 cm. by 2 cm, although the ovuliferous scales are still quite small. At this stage the most characteristic feature of the ovule is the flange-like expansion of the integument around the micropyle. It is shallowly notched at places, with, in some cases, a deeper notch on the side towards the median axis of the scale. This wide flare is not so striking, although obvious enough when viewed from the adaxial side, as shown in Pl. 2, 4, because the micropylar neck bends downwards a little, the cone itself standing rigidly erect. The flare is seen best when the cone is cut across and viewed from below. The whole flare is then exposed facing downwards and slightly outwards, as shown in Pl. 1, 5. The young nucellus lies slightly deep, but may be seen if the lie of ovule is such that the micropyle can be viewed vertically. It is shown in the ovule on the left, but appears there higher than it really is. The flange is stigmatic in function, and grains can be found on it at any part, but it cannot be as adhesive all over as other stigmatic structures, since the grains fall away from its periphery rather easily. The pollen is thus more commonly found well down towards the micropylar opening, as if the flange functioned rather like a conducting funnel. This is shown in Pl. 2, 5 and 6. The latter drawing is one of an ovule dissected to show the relatively deep position of the nucellus, and the lie of *some* of the grains. Pollen, of course, may be found much higher up, and sometimes lower down, occasionally a grain has even been seen

practically lodged on the nucellus itself. The grains are winged and float well in water. It was thought at first that the position of such grains in the micropyle might be associated with a fluid secretion, somewhat as in *Pinus*. Considerable attention was given to this point, particularly as a fluid secretion was actually seen in the micropyle in occasional ovules. It gradually became clear that any such secretion was not a normal feature. It occurred rarely, showed no tendency to withdraw into the tissue, and could become so thick and mucilaginous that pollen grains could not move in it. The secretion, when it did occur, seemed to be associated with degeneracy in some ovules and in others with local punctures made by a large aphid which often infested the cones. Punctures made anywhere, even on the scales, produced similar drops of secretion from the turgid tissues. This account has been held up to permit the examination of material over several seasons in order to decide this point. It may be further noted here that, in general, the wings or bladders of the pollen in *Abies* are relatively small in proportion to the size of the grain (cf. also Wodehouse, 1935, p. 263).

Hutchinson (1915) has already referred to a very interesting feature in the development of *Abies* based on *A. balsamea*. The pollen grains lie dormant for four or five weeks, but, when germination has begun, growth of the tubes followed by fertilization is astonishingly rapid. "Although the rate is difficult to determine, it is believed that the passage time of the pollen tube does not exceed two days, and probably may be measured in hours." This seems to be a unique phenomenon in conifers, although almost certainly a characteristic of all species of *Abies*. In *A. Nordmanniana* the pollen dormancy is extended to seven or eight weeks, germination not taking place in the grains until some time in the last week of June. In the meantime the cones, scales, and ovules have increased considerably in size. At pollination the cone was approximately 5 cm. long, the scales 3 5 mm. wide, and the ovule, including the flare, 1·4 mm. long; but now the corresponding measurements would be roughly 15 cm., 3 cm., and 0·8 to 1 cm. The micropylar flare is little changed, and can still be clearly seen as a small appendage at the top of the ovule. Apart from increase in size the only marked changes are that the original bend in the neck of the ovule becomes more distinct, the tissues of the neck thicken, particularly on the adaxial side, and, principally due to pressure against the axis as the ovule swells, the adaxial side of the flare is folded over the micropylar opening. The micropyle thus forms a short inclined tube. The nucellus, which develops a shallow depression at its summit, is very closely approached to the lowermost grains, or may even make contact with them in some cases. It has probably grown up a little to this position, although only very slight growth would be needed. All these features are shown in Pl. 2, 7, which is drawn, rather diagrammatically, from a sectioned ovule. Descriptions of intermediate stages and of the form changes of the whole ovule, as well as many other points of structure not essential to the consideration of pollen behaviour, are omitted for reasons obvious at the time of writing (1942).

The gametophyte, which in mid-June was still only in the free-nucleate

stage, is now (the last week of June) almost ready for fertilization. The archegonia are well developed; only the cutting-off of the ventral canal nucleus and the subsequent rapid maturing have to take place, although the pollen grains have not yet germinated. The succeeding days are the critical days. The actual dates of pollen germination and of fertilization probably vary a little from year to year and from tree to tree. Thus in 1936 no grains had germinated by June 30th, but on July 5th stages from fertilization to tiered proembryos were to be found. On which day in July germination first began was not determined, but it may well have been July 3rd or 4th. On the other hand in 1939 pollen grains had germinated in many ovules by June 30th, although fertilization had not taken place in any one examined. It is hoped to examine this point more accurately in the near future; the general outlines of the phenomena are, however, clear enough. In relation to this a further small change takes place in the nucellus. At some time early in these critical days the tip of the nucellus elongates a little, pushing up in a sharp wedge form among the pollen grains. Possibly the bend on the micropylar neck determines the wedge shape, but it results in bringing the nucellar depression in contact with some of the grains if contact has not already been made. Pl. **2**, 8 shows the appearance at this stage. For clarity only one grain is drawn on the depression, but many grains may be found on it. Pl. **2**, 9 shows a face view of the nucellar tip as seen on a plane at right angles to that of the last figure. Other pollen grains very close to but not in actual contact with the nucellus may also germinate, sending down short tubes which again penetrate the nucellus through the now oblique depression. In Pl. **2**, 10 and 11 the apex of the nucellus is shown in side and face view, respectively, with such a short tube growing in. Shortly after this the continued thickening of the neck and shoulders of the ovule encroach on the nucellar tip, crushing it and closing the ovule. By August it shows merely as a brown crushed lamella with the remnants of the grains and pollen tubes squeezed flat in it.

Reference to the behaviour earlier described for *Cedrus* (Doyle and O'Leary, 1935 b) shows a certain broad similarity to that here described for *Abies*. Pollen is caught in both on a spreading stigmatic flare, and upward growth of the nucellus brings it close to the grains, final contact being made by a special apical extension. In *Cedrus*, however, the form of the micropylar area is different, the nucellar function is more dominant, and the pollen tubes grow at a more normal rate. *Abies* thus can be counted as showing still another type of pollination mechanism, which is, however, closely related to that of *Cedrus*.

Strasburger's (1871) statements that no marked changes take place in the micropylar area, and that the micropyle remains open long after pollination, are thus correct; but any comparison with *Larix* is so far from the facts that it is hard to see how any such comparison could ever have been made. Likewise Goebel's (1933) inclusion of *Pinus* and *Abies* in the same pollination class is inadmissible.

(B.) OTHER SPECIES OF *ABIES*. The young ovules in *A. homolepis*, *A. koreana*, *A. nobilis*, and *A. Webbiana* are on the whole similar to those of *A. Nordmanniana*. In *A. homolepis* the body of the ovule projects a little beyond the scale and continues into a much longer micropylar neck, which is very sharply bent downwards almost at right angles. The nucellus is thus deeply sunk and must be inaccessible to air-borne pollen. Unfortunately, as the tree available bore very little pollen, the details of the behaviour could not be followed, but they would probably merely illustrate an extreme example of the type already described. If opportunity offers it is hoped to follow this point also.

A. koreana, however, was followed almost as fully as *A. Nordmanniana*, from which it differs slightly. The micropylar neck is a little longer and slightly more bent, so that, as the ovule swells, its anterior end becomes flattened against the cone axis, and the adaxial side of the micropylar area gets closely pressed against the abaxial side. The micropylar canal is thus much longer; the pollen grains caught by the flare are, except those at the extreme edge, more definitely enclosed; and those grains on the adaxial side of the flare are brought nearer to the nucellar tip, occasionally even being pressed into it. The general appearance in section, at a date about midway between pollination and fertilization, is shown in Pl. 3, 12. The flat anterior end at right angles to the side is very characteristic. Pollen grains may be found on the nucellus or anywhere along the canal and even out at the integument edge. Just before pollen germination the nucellus presses obliquely into the canal, as in *A. Nordmanniana*, but in *A. koreana* the grains at a distance germinate more freely. They have been found germinating from the outer edge of the micropylar canal, though the tubes from these probably do not reach the nucellus. Pollination takes place in the third week of April, and fertilization about mid-June or a little earlier.

Although the behaviour throughout the genus *Abies* is probably fairly uniform, it would seem advisable, in view of the differences, inconsiderable though they be, shown by the few species examined, that a fuller survey of variations within the genus should be made. Some of the species might well exhibit behaviour or structure closer perhaps to that of *Cedrus* or *Tsuga Pattoniana* than to the *Abies* type here described.

PICEA.

Pollination phenomena in this genus have been referred to by Strasburger (1871) and by Scott and Brooks (1937). Strasburger says that *Picea vulgaris* (= *P. excelsa*) resembles *Pinus* in the structure of the ovule and in the possession of two long prolongations of the micropylar edge. Between these the pollen grains pass to reach the rather deep depression at the top of the nucellus; but how this is achieved is not suggested. As far as it goes this account is correct, but it is obviously rather inadequate. Scott and Brooks, on the other hand, give no account of the structure of the integument around the

micropyle, but make the following statement (p. 287): "The integument opens widely, leaving an open passage through the micropyle leading down to the apex of the nucellus. At the same time a small quantity of fluid is secreted within the micropyle on the surface of the nucellus. Some of the pollen grains carried by the wind pass between the ovuliferous scales, and come to rest on the edge of the integument. Another change now takes place; the micropyle closes, the lips of the integument bending inwards and bringing the pollen grains into contact with the top of the nucellus, to which they adhere owing to the liquid which has been secreted." This account is correct, although inadequate, in respect of a fluid secretion, but the latter part is not based on any species of *Picea* available, and, in so far as it purports to describe the behaviour of the common spruce, is definitely wrong.

Several species of *Picea* were available. The account here given describes the behaviour in the first place in *P. excelsa* and then in P. *orientalis,* as these two show marked divergence in their pollination mechanism. A few notes on some other species of *Picea* are added.

(A.) Behaviour in *P. excelsa.* The behaviour in this species resembles that already described for *Pinus* by Doyle and O'Leary (1935 a), to which paper further reference may be made if necessary. The appearance is shown in Pl. **3**, 13 of the whole ovule as seen in adaxial view, and in Pl. **3**, 14 of the micropylar area as seen in a cone cut across and viewed from below. The neck of the ovule is a little longer than in *Pinus silvestris,* and the integument is more evenly two-lipped than in that species. The view in Pl. **3**, 14 brings out (cf. also Plate **2**, 5 for *Abies*) the manner in which the expanded integument areas project into the spaces between the scales to catch the air-borne pollen, whose movements in the air currents are to a large extent determined by the form and lie of the scales, as well as by the shape and direction of the cavities formed between them. This aspect of pollination in such cones as these is important, but has been so well described by Strasburger (1871) and others of the older authors referred to by him that no further comment is needed on it here. The edge of the integument, on the side away from the scale axis, rather evenly joins up the bases of the two stigmatic flaps, but on the inner side the edge is interrupted by a definite slit somewhat shorter than that in *Pinus.* This is also shown by Pl. **3**, 15, in which the ovule is drawn in a more strictly abaxial view. The nucellus is low and does not show without dissection, but this feature is seen in Pl. 2, 16, drawn from an ovule dissected shortly after pollination. The stigmatic flaps in nature are usually a little closer together than appears in Pl. **3**, 14. They have been splayed out a little in the drawing to emphasize their form and appearance; but, otherwise, these ovules show the common type. In some cases the slit is a little deeper, or the stigmatic flaps may be narrower and drawn out into longer processes, in which cases the ovules resemble those of *Pinus* somewhat more closely, although always quite distinct.

As in *Pinus* pollen is caught on the stigmatic flaps. A fluid is secreted, filling the space above the nucellus and welling out beyond the micropyle as a

large drop held by the two flaps and its own surface tension. The pollen wets easily and passes into the fluid, which is then fairly rapidly reabsorbed. The wings of the grains are relatively large, and orientate the floating pollen as in *Pinus*. The top of the nucellus shows a marked depression, the Einsenkung referred to by Strasburger (1871). This is developed before pollination, but becomes deeper after the deposition of the grains as is shown in Pl. **3**, 16. The edges of the depression may later grow up a little, partly embedding the pollen, the whole giving the impression of a small pollen chamber. As the grains are never found elsewhere than in the depression, it may be suggested that the reabsorption of the fluid is a function of this region.

Unfortunately it was not possible to keep the common spruce under the same continued observation as the common pine. Young trees with readily accessible cones were not available, and in older specimens cones are commonly carried towards the top of the tree, perhaps 60 ft. up or higher. Also young pine shoots carrying cones keep for a long time quite fresh in water. Indeed in the case of the pine the mechanism was first suggested from many laboratory observations before it was put to the test in the open. Spruce cones visibly deteriorate in less than a day under such conditions, so that observations were more rushed and less satisfactory, but we are quite satisfied that in general the mechanism is similar in both cases. As far as could be judged, however, from the more limited data available from laboratory and field it is probable that the action in *Picea excelsa* is not quite so precise as in *Pinus silvestris*. Thus the secretion may exude at any time once the ovule is mature, it may be found during the day hours, and is thus not a strictly night phenomenon. The exudation also is a more massive drop, and, if secretion takes place before pollination, the pollen may be more frequently caught in it than is the case in the pine. The reabsorption, while rapid, is less spectacular, requiring probably twice as long, while in some cases it is not quite complete, so that a little of it may be left to dry out on the nucellus, or to be very slowly dehydrated by absorption, leaving the grains embedded in a small sticky mass.

Strasburger's (1871) account, therefore, is correct but inadequate. The folding in of the integument, described by Scott and Brooks (1937), does not seem, however, to have any foundation in fact. Actually, after pollination, the stigmatic flaps come closer together, perhaps bending round one another a little, but gradually drying up and fading. Closure of the micropyle is effected by ingrowth and division of the cells of the neck which thickens as in *Pinus*.

Reference was made in the paper on *Pinus* (Doyle and O'Leary, 1935 a) to the fact that most of the drawings in the older authors of the common species in the Pinaceae showed little detail of micropylar structure. It is worth recording that the drawing by Richard (1826, Pl. **15**, 4) of *Picea excelsa* is very satisfactory. It is here reproduced as a text-figure, and, though simpler, obviously closely resembles the drawings of this species in this paper. We are indebted to M. Y. Orr, F.L.S., of the Royal Botanic Gardens, Edinburgh, who kindly arranged to have the drawing copied for us from the original work.

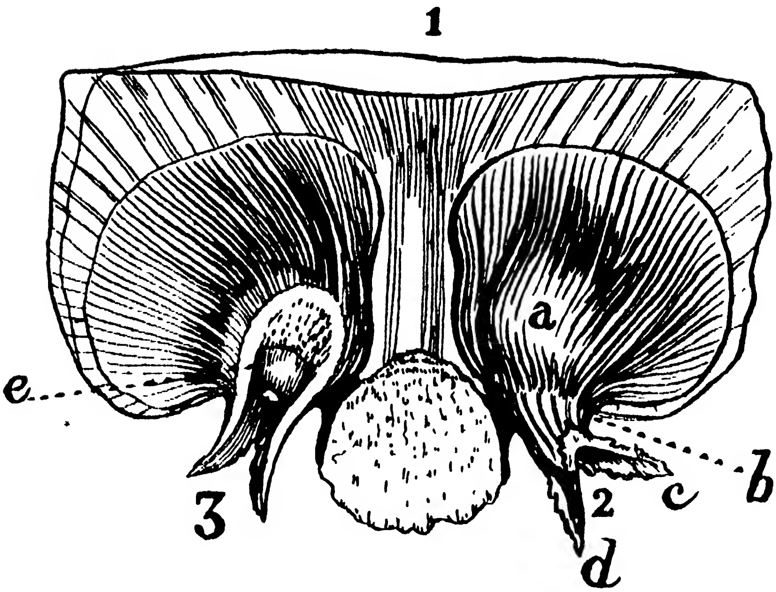

FIG. 1.—*Picea excelsa.* Scale and ovules at pollination (after Richard, 1826).

(B.) BEHAVIOUR IN *PICEA ORIENTALIS.* This species shows a characteristic which, so far as is known, is unique in the genus *Picea.* The ovulate cones leave the bud inverted, and, when mature for pollination, hang more or less vertically downward—Pl. 3, 18. In other species of *Picea* the cones stand erect when young, only turning down later as they grow older, and this is usually given as a character of the genus—thus Pilger (1926) "♀ Blüten aufrecht." Although *P. orientalis* is a fairly common tree, introduced into Britain as far back as 1839 and represented by good specimens in most collections, no record of this erect position of the young cones has yet been found in any systematic work.

This inverted lie of the cone at pollination bears directly on the pollination mechanism in this species. It was pointed out in the paper on the pollination of *Pinus* (Doyle and O'Leary, 1935 a) that the *erect* position of its young cones may be related to the flotation effect of the wings of the grain. In such cones the ovules are inverted, nearly though not quite vertically, so that the pollen floats up the fluid in the micropylar canal with the germination furrow facing the nucellus. The erect position of the young cones of *Picea excelsa* may have the same relation. If so it is to be expected that pollination behaviour in *Picea orientalis* should, in some important respects, differ from that in *P. excelsa.*

The structure of the integument round the micropyle is shown in adaxial view in Pl. 3, 19. The ovule is rounded, contracting fairly abruptly to a longish neck which is continued as two stigmatic flaps. There is a short slit on the abaxial side. These flaps differ from those in *Picea excelsa* in being much thicker and more succulent, especially at their base. As a result the micropylar

opening is more slit-like than circular. This is well seen looking down on the ovules when the cone is cut across, and is shown in Pl. 3, 20, though here also the flaps have been splayed out a little. The micropylar slit sometimes looks as if closed by the basal swelling of the two lips, but usually a small central area remains more open.

The pollen also shows a special character. Most winged grains, as in *Pinus*, other species of *Picea, Cedrus, Abies*, etc., float in water; wingless grains, such as those of *Larix, Cupressus, Taxus*, etc., sink; but *Picea orientalis* has winged grains which, nevertheless, sink. Its grains are shown in Pl. 3, 21, for comparison with those of *P. excelsa* in Pl. 3, 17. It can be seen that the grains of the former are smaller on the whole, and have relatively much smaller wings or bladders. When blown on to water the grains first float on the surface, and are not quite instantaneously wetted. When wet most of them gradually sink, but the factors determining this need further investigation. There is, however, some evidence to show that watery fluids can penetrate the wing bladders, which become more translucent as they fill with water, the grains thus losing their buoyancy rapidly. This, however, is a property of fresh pollen only; pollen, dried and kept for some time, may float for quite long periods, up to 48 hours in some cases. Again it is hoped to examine this problem more fully, but for the present it suffices that up to 90 per cent. of the fresh grains do sink.

Pollen is shed usually in the second week of May, and is caught by the stigmatic flaps. As in the case of *Pinus silvestris* and *Picea excelsa* the young ovules are in a position to catch pollen before they are fully mature, although the interval may be short. As pollen is plentiful, this early reception is common, although naturally there may be considerable variation depending on the position of the cones in relation to the liberated pollen and the like. When mature, however, the ovule of *Picea orientalis* also secretes a fairly copious fluid, which wells out through the micropyle and lies between the stigmatic flaps. Since the cones are inverted the ovules, and particularly their micropylar parts, are pointing upwards. The pollen grains caught on the stigmatic surfaces are wetted and drawn under the surface of the fluid. They float there for a few minutes, and then sink, passing through the small central channel to reach the neighbourhood of the nucellus. Retraction of the exudate follows, but slowly, needing an hour or more instead of the 10 or 20 minutes taken by the common pine or spruce. That the retraction, slow though it be, is definitely due to the presence of pollen is shown by the fact that the exudation may be continued for a couple of days in unpollinated ovules under identical laboratory conditions. In the laboratory, also, if too many grains are introduced into the fluid some may jam in the narrow opening, and these have often been seen to germinate there. This is quite rare in the field, but has been seen occasionally; such tubes make little progress. Commonly, however, when many grains are bunched on the nucellar top several are not in contact with it, they may be several diameters away. These germinate and successfully reach the nucellus. A dissected ovule with pollen on the nucellus is very similar to one of *Picea*

excelsa, and Pl. 3, 16 would do equally well for *P. orientalis*. Germination of the pollen is rapid, and in both species well developed pollen tubes can be dissected out within a week after pollination.

The behaviour in *Picea orientalis* adds another to the pollination types in the *Pinaceae*.

(C.) Other species of *Picea*. It cannot be said without a complete survey of the genus whether the type shown in *P. orientalis* is found in any other species, but it is definitely not shown by any available. The following species, at least, in addition to *P. excelsa*, have young cones erect and pollen which floats in water for 24 hours or longer:—*P. alba, brachytila, likiangensis, omorica, polita, rubra, sinensis, sitchensis*, and *Wilsoni*. A fluid exudate reabsorbed after pollination has been observed in the following:—*P. brachytila, omorica, likiangensis*, and *sitchensis*. Presumably all spruces with young cones erect resemble *P. excelsa* more or less closely in their behaviour; but many minor differences exist, and each varying structure may have its own detailed effect on the pollen behaviour. Thus the micropylar form in *P. omorica* and *P. sitchensis* resembles that in *P. excelsa*, but in the Sitka spruce the body of the ovule lies more horizontally, the two stigmatic extensions pointing downwards and markedly at right angles to the plane of the ovule. They are longer and thinner than in the common spruce. In this species the retraction of the fluid after pollination was rapid until, in its withdrawal, it reached the level of the rim of the micropyle; the subsequent much slower reabsorption was, however, not properly studied. In *P. brachytila* the whole ovule is straight, with no flexion of the flaps, and lies at about 45° to the vertical. Only the two flaps, and frequently only part of these, project beyond the scale. A wide micropylar opening is left between the stigmatic extensions, and into this the nucellus has grown at pollination time, completely blocking and even extending a little beyond the micropyle rim. Such an ovule is drawn in adaxial view in Pl. 3, 22; and in Pl. 3, 23 is shown the arrangement seen in the cone cut across and viewed from below. The pollen grains ultimately lodge on the shallow depression there shown, although the grains are not outlined in the figure. Reabsorption of the exudate was quite rapid in this species, taking only about ten minutes. If unpollinated ovules were kept for 24 hours, the exudation drop became steadily larger, but still reacted to the inclusion of pollen. If left still longer before introducing pollen no reaction or only a feeble one was obtained. The same phenomenon has been observed in *Pinus*.

Clearly then there is a fair degree of variation within the genus, and a proper examination of a larger number of species might yield interesting results, especially perhaps among those of the more recent introductions from China, whose systematy is still unsettled. Thus among specimens in the Botanic Gardens at Glasnevin, all included under *Picea likiangensis* and its varieties, there are possibly two different types of ovular structure. Presentation of further details on these and other points already raised in this paper must await the opportunity of fuller observations.

PSEUDOLARIX AND KETELEERIA.

No coning specimen of *Keteleeria* was available. Accessible coning material of *Pseudolarix* was also not available, and, from material sent from Kew, it was clear that without readily accessible material which could be kept under regular personal observation no final conclusions could be made on this genus. The young ovule resembles in general the *Abies* type, and pollen can later be found on the nucellus, but no suggestion can yet be made as to how it reaches this position. It is hoped that some other botanist, to whom coning specimens of these two genera are available, will help to complete these observations on pollination behaviour in the Pinaceae.

Certain general conclusions could be drawn from the data given in this and the preceding papers. It is proposed, however, to deal with these conclusions in a separate later communication.

Summary.

1. Observations are recorded on ovular structure in relation to pollen reception and pollen behaviour in certain members of the Pinaceae not already described in earlier papers.

2. Pollination behaviour in most species of *Picea*, except *P. orientalis*, is of the type earlier described for *Pinus*, although some variation in detail is shown in different species.

3. In *Picea orientalis* the cones hang vertically downwards at pollination. The pollen grains sink in the pollination fluid, even though winged, and thus reach the nucellus of the more-or-less upright ovules. The wings of the grain are relatively smaller, and the stigmatic part of the integument more developed than in other species of *Picea*.

4 In *Abies* no fluid is secreted at pollination. Pollen is held deep in a funnel-shaped stigmatic expansion around the micropyle. The nucellus grows up to make contact with the grains, which then germinate after lying dormant for 5 to 8 weeks. Again, some variation is shown in different species.

5. In *Tsuga Pattoniana* the pollen grains are winged, and its pollination mechanism differs from that earlier described for other species of *Tsuga*. Pollen is caught anywhere on the surface of a spreading micropylar flare, the tubes growing over the stigmatic surface to enter the nucellus tip, which is freely exposed.

6. The conclusions which may be drawn from the data given in this and preceding papers will be discussed elsewhere.

Explanation of Plates.

[All drawings made by Ann Kane. Unless otherwise stated all × 18 approx.]

Plate 2.

1–3. *Tsuga Pattoniana.* **1.** Adaxial view of ovules at pollination. May 17. **2.** Abaxial view of same. **3.** Adaxial view of two ovules about 5 weeks

later to show pollen tube growth, the nature of the stigmatic zone, and the nucellar tip. June 20.

4–11. *Abies Nordmanniana.* **4.** Adaxial view of ovule at pollination. May 8. **5.** Ovules as seen in cross-section of the cone to show nature of the stigmatic micropyle area. May 8. **6.** Ovule dissected to show position of the nucellus and some of the grains. May 8. **7.** Ovule as seen in median section at later date to show position of some of the dormant grains and apex of nucellus. June 30. **8.** Ovule as seen in median section a few days later to show oblique upgrowth of nucellar tip. July 5. **9.** Another view of the same stage in plane at right angles to last fig. **10** and **11.** Side and face view of nucellus at same stage to show growth of short tubes from grains very close to it. (Figs. 7–11 rather diagrammatic.)

PLATE 3.

12. *Abies koreana.* Ovule as seen in median section shortly before pollen tube growth to show form of micropylar canal and position of nucellus. May 26.

13–17. *Picea excelsa.* **13.** Adaxial view of ovule at pollination. May 15. **14.** Micropylar region of ovules of same stage as seen in cross-section of cone. **15.** Same in abaxial view. **16.** Ovule dissected to show position of nucellar apex with grains in the depression. May 22. **17.** Pollen grains × 60.

18–21. *Picea orientalis.* **18.** Cone at pollination hanging down. May 8. Natural size. **19.** Adaxial view of ovule. May 8. **20.** Micropylar stigmatic region as seen in cross-section of cone. May 8 **21.** Pollen grains × 60.

22, 23. *Picea brachytila.* **22.** Adaxial view of ovule to show position relative to scale, the form of the stigmatic projections and the slightly protruding nucellus. May 23. **23.** Micropylar region at same stage as seen in cross-section of cone.

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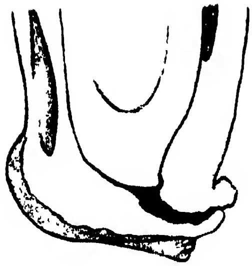
PLATE 2.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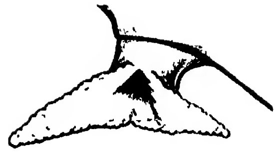
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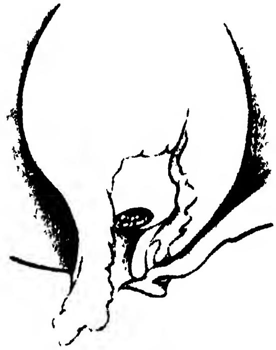
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The first mention in the literature of the examination of the lichen *Lecanora parella* appears in a paper by Schunck (Annalen., 1845, **53**, 257). Schunck isolated from the lichen lecanoric acid, a new acid which he called parellic acid, and some fatty material. It would appear, however, from the investigations of Hesse (J. Prak. Chem., 1906, **73**, 157) that Schunck was mistaken in the identification of his lichen, and that what he had in hand was in fact *Pertusaria lactea.* Nyl., a lichen which in the past has been wrongly classified by botanists as a form of *Lecanora parella.* Both Hesse (*loc. cit.*) and Zopf (Annalen., 1902, **321**, 40) examined the lichen *Pertusaria lactea.* The former found in it lecanoric acid, an acid m.p. 289° C. which he called ochrolechiaic acid, as he first isolated it (J. Prak. Chem., 1902 (2), **65**, 561) from genuine *Lecanora* (*Ochrolechia*) *parella,* and which is undoubtedly identical with the so-called parellic acid of Schunck, and traces of a third acid, which Hesse calls parellic acid, and which we now know to be identical with psoromic acid first isolated by Spica from *Squamaria crassa* (Gazzetta, **12**, **431**). Zopf (*loc. cit.*) isolated from the lichen lecanoric acid and an acid m.p. 283° C. which he calls variolaric acid, as the lichen was formerly called *Variolaria lactea.* Zopf states (Ann., 1904, **338**, 58) that variolaric acid and ochrolechiaic acid are identical, though he at first confused it (Ann., **284**, 128) with the psoromic acid of Spica. Hesse (J. Prak. Chem., 1902, **65**, 561) examined genuine *Lecanora parella,* and found no trace of lecanoric acid, but ochrolechiaic acid was present in small amount.

The present investigation of the Irish lichen which was identified for us by Dr. P. O'Connor, Natural History Section, National Museum, confirms this observation of Hesse: in addition we have identified mannitol as the sugar alcohol present. It is an optional matter as to whether the acid in *Lecanora parella* is called ochrolechiaic or variolaric acid; we prefer to adopt the latter term.

The properties of variolaric acid were first described (as parellic acid) by Schunck (*loc. cit.*), who noted its acid reaction towards litmus, and that it

crystallises with water of crystallisation, the amount varying with the conditions. He noted that it could be regenerated from solution in cold caustic potash, but that warming with aqueous potash produced a change, a precipitate being formed only slowly on acidification; he also noted that boiling with barium hydroxide gave a precipitate of barium carbonate, a barium salt remaining in solution which on acidification and extraction with ether gave a product which, like the original acid, gave a violet coloration with ferric chloride. Schunck also prepared the copper and lead salts of variolaric acid, and analysed the original acid, and its hydrates as well as its lead salt. Zopf (Ann., 1902, **321**, 40) amplifies Schunck's description of the properties of variolaric acid. He states that it melts at 285° C. with browning and gas evolution; that it is soluble with difficulty in ether, chloroform, and benzol, and with some difficulty in boiling alcohol; that it gives a colourless solution in sodium or potassium hydroxide, sodium carbonate, or ammonia; that it is not so readily soluble in sodium bicarbonate, and that it gives a colourless solution in sulphuric acid which turns a sherry colour on warming. Zopf also says that in solution in cold potash it is decomposed with evolution of carbon dioxide, and that the acid formed in the process melts at 252° C. and is readily soluble in alcohol, gives a violet reaction with ferric chloride, and an orange red, not blood red, colour with bleaching powder. Hesse (J. Prak. Chem., 1902, **65**, 561) confirms the observations of Schunck and Zopf, and notes that the acid is less soluble in alcohol than psoromic acid; Hesse, however, states (J. Prak. Chem., 1906, **73**, 1152) that the transformation of the acid on standing in potash solution is *not* accompanied by liberation of carbon dioxide, and in this respect our own results support Hesse. Hesse named the transformation product ochric acid, and describes it as melting at 230° C. with evolution of carbon dioxide. Hesse also states that ochric acid with bleaching powder gives a yellow colour destroyed by excess of bleach, a violet colour with ferric chloride, and, with sulphuric acid, a solution turning gradually yellow—statements which our observations support.

For variolaric acid Schunck found for the material dried at 110° C. C = 60 70 per cent.; 61 84 per cent.: H = 3·36; 3·42, and proposes the formula $C_{21}H_{14}O_9$. Hesse, on the other hand, gives the figures C = 62·54: H = 3 64, and proposes the formula $C_{22}H_{14}O_9$. We confirm that variolaric acid contains no methoxyl, but our analytical figures differ from those of Schunck and Hesse. We obtained for the material dried in vacuo at 105° C., C = 61·2; 61·3: H = 3 38 and 3 39. The material was not sufficiently soluble in camphor for a molecular weight determination. We propose the formula $C_{16}H_{10}O_7$. (C = 61·15: H = 3 39.) The alternative formula $C_{18}H_{12}O_8$ (C = 60·67; H = 3·37) is less in accordance with the reactions of the acid though it is close to psoromic acid, $C_{18}H_{14}8_8$, with which variolaric acid has, as regards its reactions, so much in common. When variolaric acid was dissolved in 10 per cent. aqueous potash and allowed to stand for a few hours, a product was obtained m.p. 221–223° C. (evolution of carbon dioxide) which was evidently the ochric acid of Hesse, and produced by the hydration of variolaric acid. The analytical data show that the product was $C_{16}H_{12}O_8$.

When variolaric was heated in 50 per cent. aqueous potash for 5 hours two products were obtained: one of these was soluble in sodium bicarbonate and had m.p. 195° C. with gas evolution; the other insoluble in sodium bicarbonate, but soluble in sodium hydroxide had m.p. 194–6° C. with no gas evolution. The product soluble in sodium bicarbonate was $C_{15}H_{14}O_7$, while the product insoluble in sodium bicarbonate was $C_{14}H_{14}O_5$. The reactions of variolaric acid with alkalies are thus in accordance with the equations:

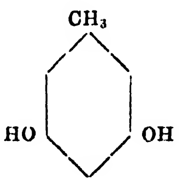
$$
\begin{aligned}
C_{16}H_{10}O_7 &+ H_2O & &= C_{16}H_{12}O_8 \\
C_{16}H_{10}O_7 &+ 2H_2O &- CO_2 &= C_{15}H_{14}O_7 \\
C_{16}H_{10}O_7 &+ 2H_2O &- 2CO_2 &= C_{14}H_{14}O_5 .
\end{aligned}
$$

These reactions recall the corresponding type of reactions in the case of psoromic acid as elucidated by Asahina and Hayashi (Ber., 1933, **66**, 1023).

$$C_{18}H_{14}O_8 + H_2O = C_{18}H_{16}O_9 \text{ (paric acid)},$$
$$C_{18}H_{14}O_8 + H_2O - CO_2 = C_{17}H_{16}O_7 \text{ (parellinic acid)}.$$

Variolaric acid forms a diacetyl derivative and with diazomethane a dimethyl derivative which is not demethylated on prolonged boiling with potash. These results indicate the presence of two hydroxyl groups, both aromatic, and the absence of a carboxylic acid group. Treated with methyl alcoholic potash variolaric acid adds on methyl alcohol forming an ester, and the resulting product on methylation with diazomethane forms a compound which now contains four methoxyl groups. These results are explained by the presence of a lactone bridge in variolaric acid, which on opening with methyl alcoholic potash forms an ester, and liberates a new phenolic group.

When variolaric acid is fused with potash two products are formed, namely orcin and α-resorcylic acid.



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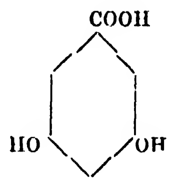
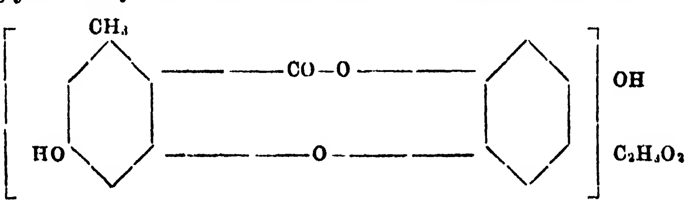
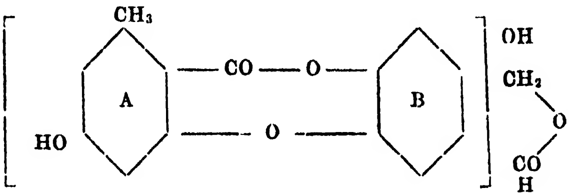


When the product obtained by the action of methyl alcoholic potash on variolaric acid is treated with chlorine, and the products of reaction subsequently reduced, there is isolated from the products 4 : 6 dichlor orsellinic ester. Accordingly we may write the formula for variolaric acid as

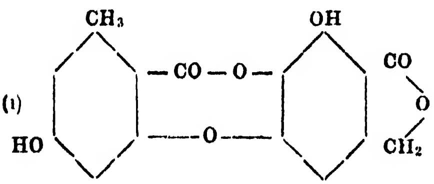


Now the following reactions of variolaric acid and its derivatives are to be noted. Variolaric acid gives a purple reaction with ferric chloride, indicating the presence in the molecule of a carbonyl group ortho to a hydroxyl: neither the acid itself nor the product obtained by opening the lactone bridge with alcoholic potash gives a blood-red reaction with bleaching powder. This latter would indicate that there is no hydroxyl in the position in ring B para to the oxygen bridge. Further it is found that the compound $C_{20}H_{20}O_8$ obtained by the action of methyl alcoholic potash followed by diazomethane on variolaric acid is soluble in methyl alcoholic potash, and that nothing is precipitated on the addition of water, but only on acidification. These results indicate the presence of a lactide group. We may write the formula now

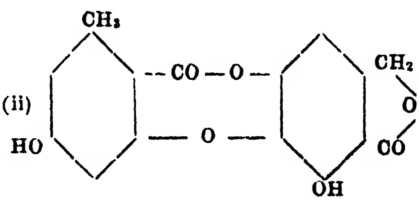


The structure of variolaric acid can then we written in any of the three forms

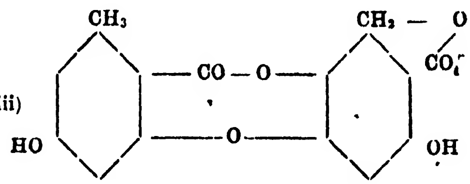
(i)



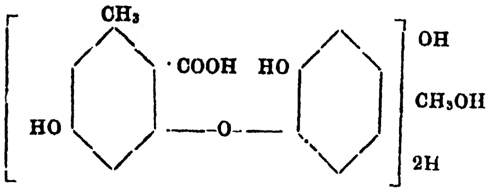
(ii)



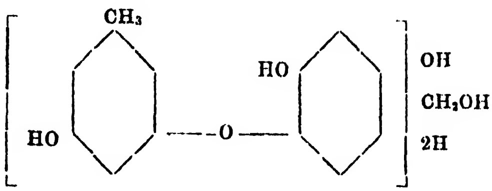
(iii)



on the ground that a carbonyl group must be ortho to a hydroxyl group to explain the violet ferric reaction of the original acid. If the a-resorcylic acid obtained in the potash fusion is coming from the B ring of the variolaric acid, then formulae (i) and (iii) are excluded, but while such is probably true, we regard it as unsafe to make this assumption because of the possibility of its arising from oxidation of orcin. However, if we turn to the product obtained by the action of 50 per cent. potash on variolaric acid we find that neither of the two products obtained gives a colour reaction with ferric chloride. For this reason it must be assumed that the carbonyl group that has disappeared in these reactions is that found in ring B, so that the products are

(iv) 

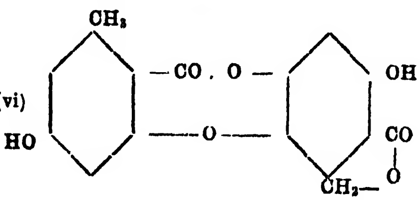
and

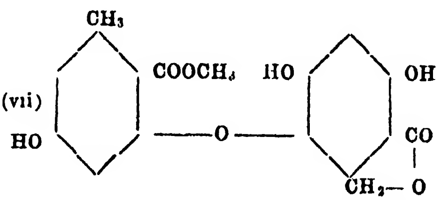
(v) 

Since neither gives a ferric reaction it follows that there is no hydroxyl ortho to the hydroxyl in ring B, so that formula (i) above is excluded.

To differentiate between formulae (ii) and (iii) we have made use of a colour reaction with 2 : 6 dichlor quinone chlorimide in a sodium borate buffer which has been employed by Gibbs (J. Biol. Chem., 1927, **72**, 649) and by Todd (J.C.S., 1940, 1208) to indicate the presence of a hydrogen para to a hydroxyl in an aromatic ring. Davidson (private communication), as a result of his work in these laboratories, has shown that the same colour reaction takes place if there be a COOH group or a chlorine group para to a free hydroxyl in an aromatic ring, but not if the acid group COOH be converted to an ester. By treatment of (iv) with the necessary amount of diazomethane the carboxylic acid group in the A ring was converted into the methyl ester, and the resulting product gave a purple reaction with 2 : 6 dichlor quinone chlorimide, thus showing a free H para to the hydroxyl in the ester of (iv). Consequently formula (iii) for variolaric acid is excluded, and the true constitution is given by the formula (ii) above.

It might be argued that the exclusion of formula (vi)

(vi) 

(vii) 

on the grounds that the product (vii) derived from it by the action of methyl alcoholic potash gives no reaction with bleaching powder is a weak one in view of the possible disturbing influence of the latent CH_2OH in ring B on the bleach reaction. However, the fact that the ester obtained by the action of methyl alcoholic potash on variolaric acid gives a blue coloration with 2 : 6 dichlor quinone chlorimide excludes the formula (vii) for the ester, and is in accordance with formula (ii) for variolaric acid given above. We exclude any arguments based on the positive imide reaction of variolaric acid only, as it is conceivable that the borax buffer, which is alkaline, might cause an opening of the lactone bridge, thus producing a carboxylic acid group para to the

hydroxyl group in ring A, which structure would of course give a positive imide reaction.

Experimental.

Extraction of the Lichen.

The lichen collected at Howth and Portrane, Co. Dublin, at Slishwood, Co. Sligo, and at Dunmore, Co. Clare, was extracted for 20 hours with acetone in a continuous extractor. A light brown crystalline product was deposited in the receiver. This was filtered off, treated twice with small quantities of hot ethyl alcohol, and once with a small amount of hot acetone to remove impurities. It was then crystallized twice from boiling 80 per cent. aqueous acetone yielding fine white crystals of variolaric acid m.p. 296° C. with decomposition and previous browning. Yield of pure material 1 per cent. by weight of lichen.

Variolaric acid is soluble on heating in 80 per cent. aqueous acetone, glacial acetic acid, and with difficulty in alcohol. It gives a purple colour with ferric chloride solution, no colour with bleach solution, and a blue colour with 2 : 6 dichlor quinone chlorimide on standing. With cold concentrated sulphuric acid it gives no colour but turns brown on heating.

Analysis: 4 030 mg. dried in vac. at 105° C. lost 0 270 mg.
(A) 3 760 mg. gave 8 440 mg. CO_2 and 1·140 mg. H_2O.
No Methoxyl found.
(B) 4·061 mg. dried in vac. at room temp. lost 0·326 mg.
3·735 mg. gave 8 390 mg. CO_2 and 1·130 mg. H_2O.
Insoluble in Camphor.
Found: (A) C = 61·2, H = 3·39; H_2O = 6 7. (B) C = 61 3, H = 3·39; H_2O = 8·0.
$C_{16}H_{10}O_7$ requires C = 61 15; H = 3 19.
$C_{16}H_{10}O_7 \cdot 1\frac{1}{4}$ H_2O requires H_2O = 6·7;
$C_{16}H_{10}O_7 \cdot 1\frac{1}{2}$ H_2O requires H_2O = 8 0.

Action of 10 per cent. aqueous potash on variolaric acid.

0 1 g. of variolaric acid was allowed to stand overnight with 5 c.c. of 10 per cent. aqueous potash, the solution turning brown. On acidification with dilute hydrochloric acid a turbidity formed which on short standing deposited brown crystals. These were filtered off and dissolved in methyl alcohol. The methyl alcohol solution was filtered and water added. On slow evaporation of the methyl alcohol in air brown crystals formed and were filtered off, m.p. 221–3° C. with frothing (on quick heating). The product effloresced in air. This compound gives with ferric chloride a colour between violet and purple, with bleach solution an initial yellow colour which rapidly vanishes, and with 2 : 6 dichlor quinone chlorimide an immediate light blue colour.

Analysis: 3·800 mg. gave 8·040 mg. CO_2 and 1·360 mg. H_2O.
0·683 mg. in 6·447 mg. Camphor. Δ = 13·6° C.
Found: C = 57·7; H = 3·97; M.W. = 300.
$C_{16}H_{12}O_8$ requires C = 57·84; H = 3·62; M.W. = 332.

Action of 50 per cent. aqueous potash on variolaric acid.

1 g. of variolaric acid was heated to gentle boiling for 5 hours in 10 c.c. of 50 per cent. aqueous potash, a hydrogen atmosphere being maintained all the time. It was cooled, poured on ice, acidified with dilute sulphuric acid, and allowed to stand overnight. It was then filtered, the filtrate extracted with ether, and the ether solution extracted with 3 per cent. sodium bicarbonate solution.

Sodium-bicarbonate-insoluble fraction.—The ether was dried over sodium sulphate and evaporated, leaving a clear colourless gum. This was taken up in a small quantity of acetic acid, and 3 volumes of benzene added to the hot solution. On standing small white crystals were deposited, m.p. 194–5° C.

This compound gives no colour with ferric chloride solution, with bleach solution a brown colour, with 2 : 6 dichlor quinone chlorimide an immediate purple colour, and with cold concentrated sulphuric acid a yellow colour changing to greenish yellow on heating. It contains no acetyl. The material may also be crystallised from warm water.

ANALYSIS: Compound dried in vacuo at 100° C.—No loss.
4·107 mg. gave 9·580 mg. CO_2 and 2·040 mg. H_2O.
0·546 mg. in 5·273 mg. camphor. $\Delta = 14{\cdot}5°$ C. (Slight decomposition.)

Found: C = 63·60; H = 5·53; M.W. = 264.
$C_{14}H_{14}O_5$ requires C = 64·1; H = 5·34; M.W. = 262.

Sodium-bicarbonate-soluble fraction.—The sodium bicarbonate solution was acidified, and gave a water-soluble oil, which was extracted with ether. The ethereal solution was dried over sodium sulphate and evaporated, leaving a reddish brown oil. This was dissolved in a little hot acetic acid, and an equal volume of benzene added to the hot solution. On short stand very small white crystals were deposited. The melting point depends on the rate of heating; slow heating 188·5° C. (frothing); rapid heating 194–6° C. (frothing).

This compound gives a brown colour with ferric chloride solution, a greenish yellow colour with bleach solution, and with 2 : 6 dichlor quinone chlorimide a light purple colour. It contains no acetyl.

A mixture with the bicarbonate-insoluble fraction melts at 183·5° C.

ANALYSIS: (A) 3·893 mg. gave 8·430 mg. CO_2 and 1·670 mg. H_2O.
(B) 3·902 mg. gave 8·360 mg. CO_2 and 1·670 mg. H_2O.
0·421 mg. in 4·012 mg. camphor gave $\Delta = 14{\cdot}0°$ C.

Found: (A) C = 59·1; H = 4·77. (B) C = 58·43; H = 4·76.
M.W. = 277.
$C_{15}H_{14}O_7$ requires C = 58·83; H = 4·58; M.W. = 306.

Methylation of the bicarbonate-insoluble compound, m.p. 194–5° C.—(*a*) With methyl sulphate; 0 1 g. of sodium-bicarbonate-insoluble compound was dissolved in 5 c.c. of 3·5 normal caustic soda. 1·5 c.c. of methyl sulphate was added, and the mixture shaken vigorously. On standing a precipitate formed, which was filtered, washed with water, and dried in vacuo. This on recrystallisation from one volume of benzene and four volumes of 60/80° petrol ether deposited clusters of thick prisms, m.p. 128–9° C.

This compound develops a faint pink colour on standing with 2 : 6 dichlor quinone chlorimide.

ANALYSIS: 3·940 mg. gave 9·620 CO_2 and 2 240 mg. H_2O.
4 401 mg. gave 7·120 mg. AgI.
Found: C = 66·6; H = 6 32; OCH_3 = 21 3.
$C_{14}H_{12}O_3\ (OCH_3)_2$ requires C = 66 2; H = 6 21; OCH_3 = 21·4.

The same compound was obtained when the methylation was carried out in boiling caustic soda solution.

(*b*) *With diazomethanes.*—0 2 g. of the bicarbonate insoluble compound was dissolved in 10 c.c. of dry acetone. Excess diazomethane was added (2 g. nitroso methyl urea), and allowed to stand corked for one day, when a slight precipitate formed. On evaporation of the solvents an oil was got which we did not succeed in crystallising.

Methylation of the bicarbonate-soluble compound, m.p. 194–6° C.—(*a*) Using excess diazomethane; 0 4 g. of the bicarbonate-soluble compound in 20 c.c. of dry acetone was treated with excess diazomethane (5 c.c. nitroso-N-methyl urethane), and allowed to stand overnight. The solvents were evaporated in air, and the residue crystallised from a mixture of benzene and 60/80° petrol ether, when clusters of pointed prisms were deposited, m.p. 108–9° C.

ANALYSIS: 3 830 mg. gave 8 750 mg. CO_2 and 2·100 mg. H_2O.
5·121 mg. gave 12 500 mg. AgI.
Found: C = 62 3; H = 6·11; OCH_3 = 32 2.
$C_{15}H_{10}O_3\cdot(OCH_3)_4$ requires C = 62·98; H = 6·08; OCH_3 = 34 26.

(*b*) Using one molecular proportion of diazomethane, 1·5 g. of the bicarbonate-soluble compound in dry acetone was treated with sufficient diazomethane to methylate only the carboxyl group. On evaporating the solvents an oil was got which was taken up in ether, the ethereal solution extracted with a small quantity of 3 per cent. sodium bicarbonate solution, dried over sodium sulphate, and evaporated, leaving a white solid which on crystallisation from acetic acid and 60/80° petrol ether gave rhombic crystals, m.p. 217–8° C.

This compound develops a purple colour with 2 : 6 dichlor quinone chlorimide.

ANALYSIS: No loss when dried in vacuo at 100° C.
4·051 mg. gave 8·880 mg. CO_2 and 1·780 mg. H_2O.
4·442 mg. gave 3·150 mg. AgI.
Found: C = 59 78; H = 4·88; OCH_3 = 9·40.
$C_{15}H_{13}O_6\cdot(OCH_3)$ requires C = 60·0; H = 5·0; OCH_3 = 9·70.

Attempts to acetylate the bicarbonate-soluble and insoluble products referred to above gave oily products which could not be obtained crystalline.

Diacetate of variolaric acid.

0·05 g. of variolaric acid was treated with 5 c.c. of acetic anhydride and 3 drops of sulphuric acid, in which it dissolved. It was allowed to stand for two hours, and was then poured into 15 c.c. water. A white precipitate formed, which was filtered off, dried, and recrystallised from 40 c.c. of methyl alcohol. On standing small rhombs were deposited, m.p. 245–6° C. with previous colouring.

ANALYSIS: 4·108 mg. gave 9 080 mg. CO_2 and 1 340 mg. H_2O.
8 340 mg. required 4 34 c.c. $N/100$ NaOH.
Found: C = 60 29; H = 3 65, CH_3CO = 22 4.
$C_{16}H_8O_7$ $2CH_3CO$ requires C = 60 3; H = 3 52, CH_3CO — 21 6.

Action of Diazomethane on variolaric acid.

0·11 g. of variolaric acid was suspended in 10 c.c. of acetone, treated with excess diazomethane (3 c.c. nitroso-N-methyl urethane), and allowed to stand for two days. The solution filled with a white flocculent solid which did not dissolve. It was then poured into a dish, and the solvents evaporated. A white residue was obtained, which was recrystallized from a mixture of equal volumes of benzene and methyl alcohol. White hairlike crystals were deposited, m.p. 260–1° C. with blackening.

ANALYSIS: 4 057 mg. gave 9·430 mg. CO_2 and 1 570 mg. H_2O.
4·523 mg. gave 6 000 mg. AgI.
Found: C = 63 37; H = 4·33; OCH_3 = 17 5.
$C_{16}H_8O_5$ $(OCH_3)_2$ requires C = 63 1; H = 4 1; OCH_3 = 18 1.

Action of 50 per cent. aqueous potash on variolaric acid dimethyl ether.

1 0 g. of variolaric acid dimethyl ether was heated in 10 c.c. of 50 per cent. aqueous potash in a hydrogen atmosphere for 5 hours. The mixture was cooled, poured on ice, and acidified, when a white precipitate formed which was practically insoluble in ether. This was recrystallised from aqueous methyl alcohol, and yielded small white crystals, m.p. 246° C. (gas evolution).

This compound gives no colour reaction with ferric chloride solution, bleach solution, or 2 : 6 dichlor quinone chlorimide.

ANALYSIS: No loss when dried in vac. at 100° C.
3·660 mg. = 8·050 mg. CO_2 and 1·500 mg. H_2O.
3·152 mg. = 3·750 mg. AgI.
Found: C = 59·95; H = 4·55; OCH_3 = 15 7.
$C_{16}H_{10}O_6 \cdot (OCH_3)_2$ requires C = 60 00; H = 4 44; OCH_3 = 17 2.

The same compound was obtained by the action of boiling 10 per cent. aqueous potash on variolaric acid dimethyl ether. The product, crystallised from methyl alcohol, had m.p. 248–9° C. not depressed on admixture with the product obtained in the previous experiment.

ANALYSIS: 4·275 mg. gave 5·580 mg. AgI.

Found: OCH_3 = 17·25.

$C_{16}H_{10}O_6\cdot(OCH_3)_2$ requires OCH_3 = 17·2.

Action of 5 per cent. methyl alcoholic potash on variolaric acid.

0·2 g. of variolaric acid was allowed to stand for 24 hours in 10 c.c. of 5 per cent. methyl alcoholic potash in the cold, when complete solution took place. It was poured into dilute hydrochloric acid, and let stand overnight to allow the alcohol evaporate in air, when a precipitate formed. This was filtered, washed with water, dried, and recrystallised from methyl alcohol and water, which yielded minute clusters of white crystals, m.p. 243° C. with decomposition.

This compound gives a violet colour with ethyl alcoholic ferric chloride solution, and with 2 : 6 dichlor quinone chlorimide a blue colour which immediately changes to dark grey-blue. When the compound is dissolved in ethyl alcohol, and bleach solution added drop by drop it gives at first a yellow colour, then brown, and on continued addition a reddish-purple colour.

ANALYSIS: 3 902 mg. dried in vac. at 100° C. lost 0·261 mg.

3 641 mg. gave 7·860 mg. CO_2 and 1 380 mg. H_2O.

4·578 mg. gave 3·110 mg. AgI.

0 392 mg. in 4·906 mg. camphor. Δ = 8·2° C.

Found: C = 58·87; H = 4·21; OCH_3 = 8·98; H_2O = 6·7; M.W. = 360.

$C_{16}H_{11}O_7\cdot(OCH_3)$ requires C = 58 95; H = 4·05; OCH_3 = 8 96; M.W. = 346.

$C_{16}H_{11}O_7\cdot(OCH_3)\cdot 1\frac{1}{2}H_2O$ requires H_2O = 7·2.

Action of diazomethane on ester, m.p. 243° C.

0 18 g. of the previous compound was dissolved in 10 c.c. of acetone, and allowed to stand corked with excess diazomethane (3 c.c. nitroso-N-methyl urethane) overnight. The solvents were evaporated in air, the residue dried in vacuum, and recrystallised from methyl alcohol, yielding long white needles, m.p. 181–2° C. The compound gives no colour reaction with ferric chloride solution. It is soluble in methyl alcoholic potash, the solution on dilution with water and removal of the methyl alcohol giving no precipitate until acidified.

ANALYSIS: 4·536 mg. gave 10·270 mg. CO_2 and 2·110 mg. H_2O.

4 230 mg. gave 10·160 mg. AgI.

Found: C = 61·73; H = 5·17; OCH_3 = 31·73.

$C_{16}H_8O_4\cdot(OCH_3)_4$ requires C = 61·85; H = 5·17; OCH_3 = 31·96.

When this experiment was repeated by letting the compound stand for 3 days with excess diazomethane the same product was obtained. M.p. 182° C., unchanged when mixed with the above compound. The same product was also obtained when the methylation was carried out with potassium carbonate and dimethyl sulphate in boiling acetone.

Potash fusion of variolaric acid.

20 g. of caustic potash pellets and 2 c.c. of water were heated to 100° C., and then 1 g. of variolaric acid was added slowly with stirring. The temperature of the melt was raised to 250° C., and maintained at that temperature for 20 minutes and then at 310° C. for 5 minutes, when the colour of the melt changed to red. The melt was cooled, dissolved in water, carbonated for 30 minutes, and then acidified with dilute sulphuric acid. The acidified solution was extracted with ether, and the ethereal solution extracted, first with 3 per cent. sodium bicarbonate, and then with 3 per cent. sodium hydroxide, and the solutions worked up as follows:—

(*a*) The bicarbonate solution was acidified with dilute sulphuric acid, extracted with ether, the ethereal solution dried with sodium sulphate, and evaporated, leaving a brown gum containing some crystals. This was boiled with benzene, and gave a dark soluble residue, which was dissolved in water, cleared with charcoal, filtered, and the aqueous solution evaporated to dryness. A white residue was obtained, which was dissolved in acetic acid, filtered, and two volumes of benzene added to the filtrate, when white plates were obtained, m.p. 230–232° C. not depressed on admixture with α-resorcylic acid.

ANALYSIS: 2·958 mg. vac dried at room temperature—no loss.
2 958 mg. gave 5 880 mg. CO_2 and 1·070 mg. H_2O.

Found: C = 54·2; H = 4·06;
$C_7H_6O_4$ requires C = 54 5; H = 3 9.

(*b*) The sodium hydroxide solution was acidified with dilute sulphuric acid, extracted with ether, the ethereal solution dried over sodium sulphate and evaporated, leaving a brown oil. This was dissolved in hot chloroform, and some drops of hot water added. On standing large platelike crystals were precipitated, m.p. 57–58° C. unchanged when mixed with orcin. This compound gave no colour with ferric chloride solution, and a blood-red colour with bleach solution.

Action of chlorine followed by reduction on the product m.p. 243° C. obtained by the action of methyl alcoholic potash on variolaric acid.

1 g. of this compound was suspended in 75 c.c. of chloroform, and treated with 41 c.c. of carbon tetrachloride containing 2 g. chlorine, and left stand corked 5 days. The chlorine and hydrochloric acid were then removed by bubbling dry air through the solution. The solvents were evaporated, leaving a greenish brown oil. This oil was reduced by dissolving it in 30 c.c. of glacial

acetic acid and adding gradually 3 g. of stannous chloride in 60 c.c. of 20 per cent. hydrochloric acid. The colour changed to reddish-brown, and a gum separated on the walls of the flask. The material was diluted with water, extracted with chloroform, and the chloroform evaporated. The residue was taken up in ether and extracted with 3 per cent. sodium bicarbonate. The ethereal solution was evaporated to dryness, and the residue distilled in superheated steam, when crystals passed over. These were collected, crystallised twice from ligroin, and once from aqueous methyl alcohol, yielding fine needles, m.p. 115° C. (corr.) not depressed when mixed with 2 : 4 dichlor o-orsellinic acid methyl ester (Nolan and Murphy, Sci. Proc. R.D.S., **22**, 317).

This compound gives a purple colour with ferric chloride and a blood-red colour with bleach solution.

2 : 6 *dichlor-para-orsellinic acid methyl ester.*

0·34 g. 3 : 5 dihydroxy p-toluic acid methyl ester was dissolved in 5 c.c. of chloroform, and treated with 7 1 c.c. carbon tetrachloride containing 0 33 g. chlorine, the whole being cooled. After standing corked for 1½ hours excess chlorine was removed by bubbling dry air through the solution. The solvents were evaporated in air, and the residue crystallised from methyl alcohol or benzene, yielding small white crystals m.p. 167–9° C.

This compound gives a dark blue colour with ferric chloride solution, no colour with bleach solution, and with 2 : 6 dichlor quinone chloromide a pink colour. The material was dried in vacuum at 80° C. before analysis.

ANALYSIS : 3 282 mg. gave 5 220 mg. CO_2 and 0 970 mg. H_2O.
5 959 mg. gave 6 780 mg. AgCl.
4 980 mg. gave 4·810 mg. AgI.

Found : C = 43·38; H = 3·3; Cl = 28·14; OCH_3 = 12 7.
$C_7H_5O_2Cl_2\cdot(COOCH_3)$ requires C = 43·03; H = 3 2; Cl = 28 28; OCH_3 = 12·3.

Alcohol extraction of Lichen.

The air dried lichen from the acetone extraction was boiled for three hours with ethyl alcohol. On filtering and evaporating the solvent a small quantity of green extract was obtained, which was dried, extracted with 50 c.c. of water, and the water evaporated, leaving a brown gum. This was washed with 35 c.c. of warm methyl alcohol. The portion insoluble in methyl alcohol was left as a brown powder, which on crystallisation from boiling methyl alcohol yielded white crystals, m.p. 162–3° C. not depressed on admixture with mannitol.

One of us (D. M.) acknowledges with thanks a grant from the Department of Industry and Commerce which has enabled him to collaborate in this investigation.

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SALMON AND SEA TROUT OF THE WATERVILLE (CURRANE) RIVER.

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Price Two Shillings and Sixpence.

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[Read 15 December, 1942. Published separately 7 April, 1943.]

The Waterville River and its Tributaries.

The Waterville River and the Sligo River are the only rivers in Éire to open for salmon fishing by means of nets and weirs on New Year's Day, the number of fish taken at Waterville in the month of January often exceeding the catch of any other month of the fishing season. The main fishery on the river, known as the Waterville Fishery, is held under an ancient charter, and consists of an old-established weir and a pool which is fished by means of a draft net. Under the provisions of an Act entitled "An Act to amend the laws relating to Fisheries in Ireland" (26 and 27 Vict. Cap. 114) all fishing weirs had to be provided with a free gap for the passage of fish, constructed in accordance with certain well-defined rules. In the case of chartered or patent weirs existing in 1863 on rivers less than forty feet wide a concession was made, and the authorities were, by Section 11 of the said Act, empowered, if they thought fit, to extend the normal weekly close time by twenty-four hours in lieu of a free gap. The provisions of this section have been availed of in the case of the Waterville Salmon Weir, and a weekly close time from 12 o'clock noon on Friday to 12 o'clock noon on the Monday following operates instead of the usual weekly close time from 6 a.m. on Saturday to 6 a.m. on the Monday following. Netting in the pool below the weir is also restricted by statute and by-law to the same period. It is also interesting to note that after their capture both salmon and sea trout are often kept alive in a fish box for several days before despatch to the London and other markets.

The Waterville River is some 500 yards long and enters the sea through a cutting in a boulder storm beach. It drains Lough Currane, a sheet of water about 3½ miles long, 2 miles wide, and up to 90 feet deep in the deepest parts. The character of the river below the Salmon Weir has changed somewhat in recent years, because at one time this portion of the river was looked upon as tidal, whereas at present it is neither salt nor is the height of water in the river influenced by the majority of tides. It is obvious, however,

that the prevailing heavy seas and their effect on the contour of the beach may have been responsible for this change.

There are numerous islands in Lough Currane, which forms one of the most attractive features of Waterville as a fishing and holiday resort. At the eastern end of the lake there are two important tributaries, of which the northern, the Cummeragh River about four miles long is the larger, and is fed by a number of lakes (Loughs Derriana, Cloonaghlin, Namona being the chief) and mountain streams, to which salmon and sea trout resort in large numbers to spawn. The mouth of the river is in a bay, of which the entrance is partly closed by small islands. A considerable number of salmon and sea trout are taken by angling in the lower stretches from about March onwards. The upper

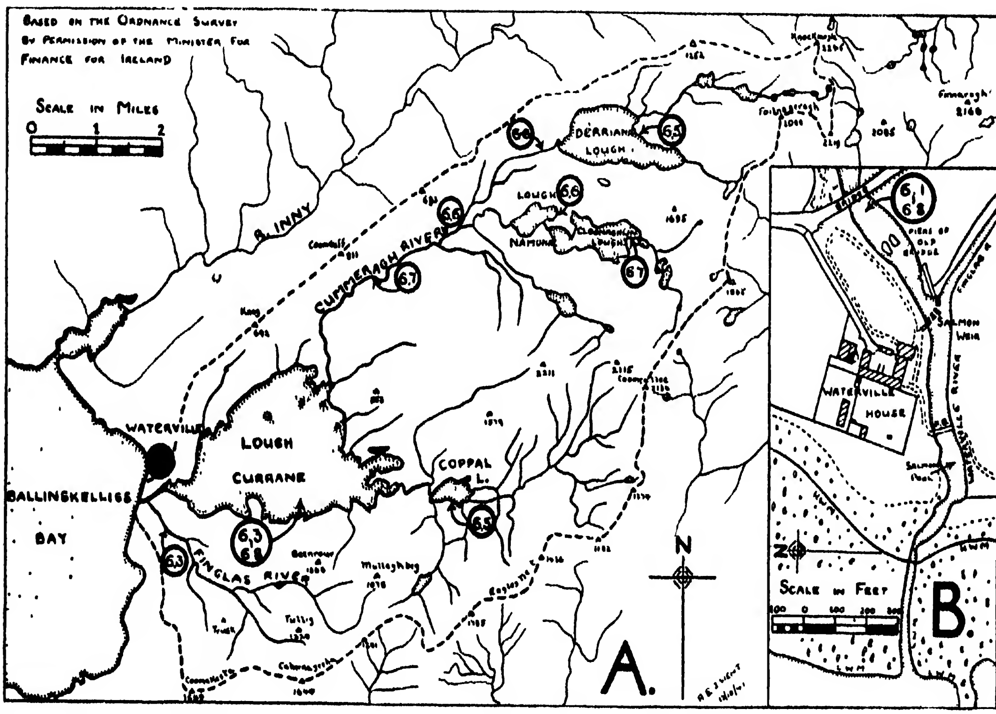

Fig. 1.—A. Sketch map of the catchment area of the Waterville River and its tributaries.

B. Sketch map of the Waterville Fishery.

(Figures inside circles indicate pH values in normal heights of water.)

reaches of tributaries of the Cummeragh River are little more than mountain torrents, especially those draining part of the southern slopes of Knockmoyle (2,245 feet high) and adjacent mountains. The other important tributary, entering Lough Currane to the south of the mouth of the Cummeragh River, is a stream about a mile long which comes down from Coppal or Isknagakiny Lough, and is frequented by a fair number of salmon and sea trout. Another tributary, the Finglas River, entering the Waterville River just below the

Salmon Weir occasionally holds salmon and sea trout during the season, the main run being of almost ripe fish after the close of the fishing season.

As the season progresses salmon entering Lough Currane from the sea tend to assemble near the mouths of the tributary rivers, awaiting favourable conditions which arise when a flood comes down after heavy rain. In consequence of this, netting in the lake has been prohibited by by-law in order to maintain the stocks of fish.

The whole of the waters of the Waterville River and its tributaries flow over rocks belonging to the Old Red Sandstone, which are overlaid in places by glacial drift. As the rocks of the river basin contain very little lime the waters of the various tributaries and lakes are either slightly acid or neutral. In Fig. 1A the pH values of the waters of selected localities have been indicated, and in Table I (see Appendix for tables) typical analyses of the waters from certain localities in the catchment area have been given. The total area of the basin of the Waterville River amounts to 46 square miles. In Fig. 1A the disposition of the Waterville River, its lakes and tributaries has been indicated.

Material.

The material used in this investigation consists of 698 satisfactory sets of scales and measurements of salmon, and 433 similar sets from sea trout captured in both the weir and net fishery on the Waterville River (see Fig. 1B). The bulk of the material was collected during the year 1941 under the guidance of one of us (T. S. B.), but a number of sets relating to both species of fish were collected in previous years by Mr. James W. Butler, the owner of the Waterville Fishery. The number of fish sampled in each month is given in the following table :—

Month.			Salmon.	Sea Trout.
January	...	...	147	
February	...	...	129	51
March	...	...	133	
April	...	...	53	163
May	...	...	62	115
June	...	...	114	96
July	...	...	60	8
TOTAL	...	...	698	433

Examination of the above table, and of confidential returns of the catch of 1941, indicates that the numbers of fish sampled were fairly representative of the total catches, save in that the runs of sea trout after the 15th July were not sampled. There were unavoidable fluctuations in the percentage of fish sampled, but by suitable arithmetical manipulation the data supplied can be used to obtain a fairly accurate idea of the incoming salmon and spring sea

trout populations. Wherever possible the weighted figures have been given in the tables.

Salmon begin to run into the Waterville River in fairly large numbers during the month of December, and continue to run in force until the beginning of August. The legal season for netting in the Waterville River is from 1st January until 15th July, both days inclusive, and it is obvious that the material will not contain samples of the runs of salmon in December or those after 15th July. As will be shown later, two age groups, namely the 1 + and 2 winters age groups, formed nearly 90 per cent. of the total catch of salmon, and in consequence the results will not be materially affected by the lack of samples from December and after the 15th July. In the case of sea trout, which normally begin to run in February or March, the absence of samples after 15th July means that the later running sea trout will be absent, so that the material is not representative of the runs of the sea trout as a whole, but may be considered a fairly adequate sample of the sea trout runs up to the end of the netting season, i.e. of the spring runs.

All fish were measured from tip of the snout to the fork of the tail, and the length was recorded to the nearest tenth of an inch.

Age and Growth of the Salmon.

Smolt Ages.

The distribution of the various smolt ages in each age group is given in Table 3. The bulk of the fish migrated as two-year smolts, and the proportion of three-year smolts exceeded that of the one-year smolts. The Waterville River resembles in this respect the Rivers Owenduff and Erne (Went, 1941 and 1942), but differs considerably from the River Shannon and Ballisodare River (Went, 1938, 1940 and 1941). The percentage of young smolts was slightly greater in the small spring fish than in the grilse.

Age Groups.

The fish whose scales were satisfactory for age determinations were divided into four groups of maiden or unspawned fish and one group of previously spawned fish usually marked "with S.Ms." (with spawning marks on the scales). The results have been given in Table 4. Grilse (1 + winters) and small spring fish (2 winters) formed together 88·3 per cent. of the total catch. Small spring fish were most abundant, with a percentage of 55 4 per cent. It is surprising that, in a river having such early runs of salmon as the Waterville River, the proportion of large spring fish is so small. Apart from the scale readings, the fact that the average weight of fish in the first four months of the year did not rise above 12·0 lb. in 1941 confirms the view that large spring fish were not numerous (Table 2). A few summer fish were taken in April, in May they formed about one-third of the total catch, and in June and July they formed over 90 per cent. of the whole catch (Table 4). Grilse were most plentiful in July, and if samples had been taken during the whole of that month the figure would have been somewhat in excess of that given in Table 5. By the close of

the netting season the runs of grilse had, however, diminished in size very considerably. The peak of the small summer fish (2 + winters) run was in June, whilst most previously spawned fish, the bulk of which had spawned for the first time as grilse, were taken in July. The best month for small spring fish (2 winters) was February. In the months of April and May the runs of fish were relatively poor.

It might be asked whether the results for 1941 can be applied to previous years. Consideration of the confidential returns of the Waterville Fishery over some ten years revealed that, in general, the runs in 1941 showed a similar distribution in time to the runs in previous years. The year 1941 was probably not abnormal in most respects, and similar conditions would appear to have prevailed over a number of years.

The previously spawned fish formed 7 2 per cent. of the total catch. One fish, out of the 44 of this group examined, had two spawning marks on its scales, whilst the remainder had one spawning mark only. Previously spawned fish can be classified on (1) "Absence," the time spent feeding in the sea between what would have been, but for the intervention of man, two successive spawnings, and (2) the age at first spawning. In Table 6 the previously spawned fish have been so classified. As is usual, the spring fish adopted the long absence habit, whilst the grilse adopted the short or very long absence habit.

Divided Migration and Return.

Table 7 gives the years in which the fish of the 1941 catches were hatched. It will be seen that in 1941 the year classes of 1938 (fish in their fourth year) and 1937 (fish in their fifth year) were the most important. From the commercial point of view, however, the 1937 year class was considerably more important than is indicated by its numerical proportion in the total catch owing to the comparatively high average weight of its members.

Size Distribution.

Table 8 gives the estimated percentage of fish occurring in each two-inch class interval. Fish having lengths between 23 95 and 33·95 inches formed 85 per cent. of the total catch. The class interval of 30 (fish having lengths between 29·95 and 31 95 inches) formed about one-quarter of the total catch in 1941.

Condition Co-efficient.

When considering the relationship between the weight and length of salmon it is usual with workers using British units of measurements and weight to adopt a formula devised by Menzies (1921) when he was investigating salmon of the Scottish Dee. The relationship, called the condition co-efficient (K), is determined from the following formula:—$K = W/(L^3 \times 0{\cdot}00036)$, where W = weight in lb. and L = length in inches. This formula gave a value approximating to unity for salmon of the Scottish Dee, which were therefore taken as a standard. Unfortunately salmon of the Scottish Dee are somewhat lighter, length for length, than fish from most of the rivers investigated to date.

It might be mentioned that if metric units are used, and a condition factor is calculated using the formula, 100,000 W/L^3, where W = weight in kilogrammes and L = length in centimetres, the results closely approximate to those given by Menzies' formula (they actually differ by 0·4 per cent. only). Despite the close approximation of the results it would seem desirable that a standard method should be adopted for calculating the relationship between weight and length in salmon and other salmonidae. The metric formula, which, unlike Menzies' formula, does not imply the idea of a standard fish, would appear to be the most convenient one for general adoption.

Some criticism might be levelled at a worker for using British instead of Metric units in scientific work. With a valuable fish such as the salmon it is obviously impossible to obtain large numbers of fish for investigation in the laboratory, and recourse has to be had to other methods of obtaining material. Usually this is done, as in the present case, by arranging for some reliable person to make collections of sets of scales and measurements, etc., of salmon taken in a fishery on the river which it is desired to investigate. It has been one of the author's experiences in Ireland that the use of metric units in the collection of such data leads to inaccuracies and obvious mistakes, and on account of this all measurements have been made with what were to the collectors the familiar British units of weight and length.

Owing to difficulties which arose from the arrangements for the despatch of the fish to the markets it was not found possible to weigh all the fish. Selections of fish were, however, weighed during each month, and the condition co-efficients have been calculated for each age group from these data. The mean condition co-efficient on Menzies' scale for each age group is given in Table 9, the mean value for all fish being 1 08. Spring fish were in better condition than the summer fish, the average condition co-efficients being 1·10 and 1·05 respectively. In this respect salmon of the Waterville River resemble those of the River Shannon (Went, 1938 and 1940). The mean condition co-efficients were, however, considerably lower than those of salmon from Irish rivers already investigated (Southern, 1928; Went, 1938, 1940, 1941, and 1942).

Average Sizes.

Details of the average lengths and weights will be found in Table 10. As only a proportion of the fish was weighed, the average weight of each age group was calculated from the average length and the average condition co-efficient. That these estimated average weights were fairly accurate is shown by the fact that the estimated average weight of all fish examined was 10·06 lb., whereas the actual average weight per fish as indicated by the confidential returns was 9·9 lb. (Table 2).

(*a*) Grilse (1 + winters). Number examined = 157.

	inches.	lb.
Minimum	22·1	3·9 (actual)
Average	25·4	5·9 (estimated)
Maximum	30 0	—

The first grilse was taken in 1941 on 29th May. The minimum length was recorded on two occasions, namely, 5th and 7th June, whilst the maximum length was recorded on 26th June.

(*b*) Small spring fish (2 winters). Number examined = 464.

	inches.	lb.
Minimum	27 0	—
Average	30 9	11·7 (estimated)
Maximum	37 5	—

The minimum length was recorded on 14th February, whilst the maximum length was recorded on 23rd May.

(*c*) Small summer fish (2 + winters). Number examined = 20.

	inches.	lb.
Minimum	26·0	5 5 (actual)
Average	31·8	11 6 (estimated)
Maximum	36·0	—

The shortest fish was captured on 13th June, and the maximum length was recorded on two occasions, namely, 23rd May and 20th June.

(*d*) Large spring fish (3 winters). Number examined = 13.

	inches.	lb.
Minimum	35 0	16 0 (actual)
Average	38·1	22·7 (estimated)
Maximum	42·5	30·0 (actual)

The minimum length was recorded on two occasions, namely, 24th January and 25th April, whilst the maximum length was recorded on 24th January.

(*e*) Previously spawned fish (with S.Ms.). Number examined = 44.

	inches.	lb.
Minimum	26 0	—
Average	34·5	16·7 (estimated)
Maximum	41·5	25 5 (actual)

The smallest fish was captured on 26th June and had adopted the short absence habit, having spawned for the first time as a grilse. The largest fish, which had adopted the long absence habit, was captured on 14th March, having spawned for the first time as a small spring fish. Average weights in this group are of little value, because it consists of a heterogeneous collection of fish having only one common character, namely, the presence of spawning marks on their scales.

Erosion.

Erosion, or more properly absorption, of the substance of the scales begins to take place when the gonads of salmon are ripening. Usually this takes place in fresh water, but it can also take place in the sea if the fish has been delayed

in reaching fresh water for any reason. In the material at the authors' disposal six spring fish showed slight erosion, none of which, however, exceeded 3° on Menzies' scale.

Calculated Lengths.

The length of each "maiden" or unspawned fish at the end of each year of its life was calculated in the usual way, from measurements of the scales, i.e. on the assumption that the growth of the scale is strictly proportional to the growth of the fish.

A. River Life.

The relationships of the parr lengths are dealt with in this section. In Table 11 the calculated lengths of the parr at the end of each winter of freshwater life in the various smolt classes are given (see also Fig. 2A). It will be seen that the most rapidly growing smolts migrated first. At the end of the first year in fresh water the one-year smolt class was, on the average, longer than the two and three-year smolt classes, the two-year smolt class being superior in length to the three-year smolt class. Similar conditions have also been described for other rivers in Ireland (Went, 1938, 1940, 1941, and 1942).

In previous publications dealing with the salmon of the Rivers Shannon, Ballisodare, Owenduff, and Erne, it was stated that smolts in these rivers fell naturally into two groups, namely those which exhibited freshwater growth in the spring prior to migration to the sea, and those which showed no growth in the spring prior to the smolt migration. In the Waterville River a similar condition was observed. If new freshwater growth is limited to one or two annuli it is often difficult to define the limits between the winter and spring growth. To eliminate this difficulty the smolts were divided into two groups, namely:—

(*a*) One group, termed type A smolts, which showed little or no new growth in fresh water in the spring prior to migration as a smolt, i.e. new growth restricted to two new annuli or less, and

(*b*) one group, termed type B smolts, containing those fish which have made considerable growth in fresh water in the spring prior to migration to the sea.

The distributions of the two smolt types in the various smolt classes are given in Table 12 as percentages of the total maiden fish. Almost three-quarters of the total catch could be referred to the two-year type B smolt class, and in this respect salmon of this river resemble those of the Rivers Owenduff and Erne (Went, 1941 and 1942).

The average lengths of fish belonging to the two smolt types in the various smolt classes have been calculated, and the results are recorded in Table 13. At the end of each year of freshwater life the type A smolts were longer on the average than type B smolts of the same smolt class, Fig. 2B. Similar conditions have already been described for salmon from the Rivers Shannon,

Ballisodare, Owenduff, and Erne, and a hypothesis was advanced to explain this phenomenon. Briefly the hypothesis is that salmon parr must attain some physiological condition associated, at least as an index, with a minimum size before the smolt migration takes place. Jones (1939), dealing with the salmon of the Cheshire Dee, raises objections to the acceptance of this view on the

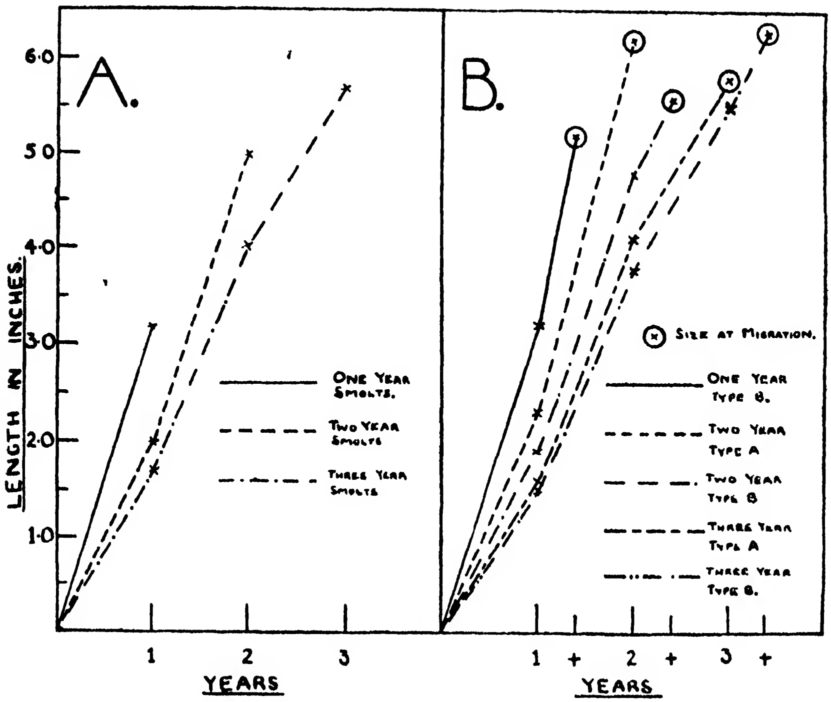

FIG. 2.—Fresh water growth in salmon.
A. Growth curves of the three smolt classes.
B. Growth curves of the different growth types.

grounds that he finds a big variation in the calculated lengths of the smolts from that river, and that some very large smolts have been observed. This criticism is based, to some extent, on a misunderstanding of this hypothesis which does not suggest that migration occurs immediately an individual reaches a certain length. Dealing as we are with biological material, it is not to be expected that the separation of migrating salmon parr would occur with mathematical precision such as is usually the case in forms of mechanical separation. If the validity of the hypothesis is assumed, it might be expected that lengths of the individual smolts, if plotted, would produce a normal curve with the mean corresponding with the value of the "minimum size." In this way the variation observed by Jones is explained to some extent.

In previous publications the calculated smolt lengths of salmon from the Rivers Shannon, Ballisodare, Owenduff, and Erne, have been given and are recapitulated in the following table for sake of convenience:—

Shannon, 1928 (Went, 1940)	... ...	5·54 inches
Ballisodare (Went, 1940)	... ...	4 87 „
Owenduff (Went, 1941)	... ...	4·9 „
Erne (Went, 1942) ...	... ...	5 6 „
Waterville	... ...	5·7 „

It will be seen that there is a considerable variation in the average smolt length in the five Irish rivers investigated. If a variation occurs in different rivers there is no reason to believe that similar variations do not occur in the different portions of a river, particularly in a large river where different tributaries run over rocks of different geological formations. This would also explain to some extent the facts observed by Jones in the Cheshire Dee.

In general no difficulty is experienced in distinguishing between sea and freshwater growth as exhibited by the scales, and therefore it is quite possible to calculate the length of each individual smolt. The average lengths of the smolts in each smolt class are given in Table 11. It will be seen that the average size rose as the smolt age increased. With an average length of 5·7 inches, smolts of the Waterville River are similar in size to those of the Rivers Shannon and Erne, but considerably larger than those of Ballisodare and Owenduff Rivers. From examination of Table 14 it would appear that no differentiation in length in fresh water occurs in the various age groups. The average smolt lengths in the two-year smolt class show little variation in the various age groups.

B. Sea Life.

The length at the end of every year in the sea was calculated for every fish with suitable scales. In Table 14 these lengths are given for the various age groups. At the end of the first sea winter the grilse have a greater average length than any other age group; this being more readily seen by comparing all grilse with the remaining age groups as follows:—

Age Group.	No. examined.	Average length at end of 1st sea winter.
Grilse	156	20 2 inches
2, 2 +, and 3 winters age groups combined ...	486	19 3 „

C. Correlation Tables.

In this section it is proposed to discuss the relationship between the growth made in the various years of life.

(1) Correlation between parr lengths at end of the first and second winters in the two-year smolt class.

Table 15 gives the correlation between the length of parr at end of the first and second winters in the two-year smolt class. It will be seen that if the fish are grouped in ascending order according to the size at the end of the first winter the increments made in the second year show a gradual increase.

(2) Correlation between smolt length and length at the end of first sea winter.

It has already been mentioned that the grilse were, on the average, longer at the end of the first sea winter than any other group. In the following discussion the grilse and small spring fish have been considered separately and Table 16 gives the correlation between smolt length and length at the end of the first sea winter in each of these age groups. The smolts which were longer than average maintained their superior length at the end of the first sea winter.

(3) Correlation between lengths at the end of first and second sea winters in small spring fish.

Table 17 gives the correlation between the lengths at the end of the first and second sea winters in the small spring fish. It will be seen that fish which were of average length at the end of the first sea winter were of average length at the time of capture (end of second sea winter). The average increment in the three groups given in Table 17 is approximately the same.

AGE AND GROWTH OF THE SEA TROUT COMPRISING THE SPRING RUNS IN THIS RIVER.

The material at the authors' disposal was obtained almost exclusively from what might be termed the spring runs of sea trout into the Waterville River and the monthly catches, estimated as a percentage of the catch in the year 1941, have been given in Table 18. In general the average weight (Table 2) decreases as the season progresses. This can be shown to be due to the fact that the proportion of maiden or unspawned fish and, therefore, comparatively light fish, increases with the progress of the season (Table 19). A small proportion (15·7 per cent.) of the total were maiden fish entering the river for the first time, whilst the remainder were previously spawned fish.

Smolt Ages.

Four smolt ages, namely, two, three, four, and five years, were observed, only one five-year smolt being noted. In Table 20 the distribution in the different smolt ages has been given. Two and three-year smolts were almost equal in proportion, and formed together over 90 per cent. of the total.

Age at Maturity and Number of Spawning Marks.

In the previously spawned fish, 28 or 7 per cent. had spawned for the first time in their first post migration winter, i.e. as whitling, 280 or 75 per cent. in the second post migration winter, and 67 or 18 per cent. in the third post migration winter. In general the older smolts tend to return after a short initial feeding in the sea, as will be seen from Table 21. The calculated proportion of the different groups in the maiden fish was as follows. First post migration summer 2 per cent., second post migration summer 45 per cent., third post migration summer 51 per cent. and the fourth post migration summer 2 per cent. There is an apparent discrepancy between the results obtained

from previously spawned and maiden fish. This is due to the fact that the young maiden fish tend to run more abundantly after the close of the netting season, and are, therefore, less liable to capture by the methods employed.

Of the 375 mature fish 119 or 31·7 per cent. had a single spawning mark on their scales, 118 or 31·4 per cent. had two such marks, 97 or 25·9 per cent. three spawning marks, 28 or 7·5 per cent. four spawning marks, and 13 or 3·5 per cent. five spawning marks. Only one of the previously spawned fish had spent more than a year feeding in the sea between what would have been, but for the intervention of man, two successive spawnings.

Condition Co-efficient.

The formula usually adopted by workers using British units of weight and length is that devised by the workers in the Fishery Board for Scotland and used by the late G. H. Nall in his numerous investigations on sea trout (Nall, 1930). This condition co-efficient is calculated according to the formula $K = W/(L^3 \times 0{\cdot}000427)$, where W is the weight in pounds and L is the length in inches. Using this formula Nall found that in good summer condition a mean value of 0·95 was obtained for fish of whitling (first post migration summer) size, and about 1·0 for larger sea trout. In the material at our disposal only a proportion of the fish was weighed owing, as was explained in connection with the salmon of this river, to difficulties experienced in the marketing of the fish. A selection of the fish was weighed, and the condition co-efficients were calculated by means of the above formula. The monthly mean value of the condition co-efficient is given in Table 22.

The mean condition co-efficient ($K = 0{\cdot}9$) is low, but this does not necessarily imply that the sea trout of this river were inferior in edible or sporting characteristics. Just as it has already been shown that there is a considerable variation in the average condition co-efficients of salmon taken in different rivers in Ireland, so with sea trout there is a considerable variation in "condition" from river to river. The spring runs of sea trout into the Waterville River would appear to be slim fish, which may be the result of a very short period feeding in the sea after spawning in the case of over eighty per cent. of the fish.

Whilst dealing with the subject of "condition co-efficient" it is worthy of note that Menzies devised the above formula on the basis of weights and measurements of sea trout from the River Forth in Scotland. Hence the sea trout of the River Forth were used as a standard as were the salmon of the Scottish River Dee. The views expressed by the authors in an earlier section of this paper in connection with the condition co-efficients for salmon may be considered to apply with equal emphasis to the sea trout.

Calculated Lengths.

It is usual in calculating the lengths of sea trout from measurements of the scales, to ignore those fish which have spawned previously. In this case, owing to the very large proportion of mature fish it was decided to measure

all fish, but it might be pointed out that the results obtained from these measurements did not differ materially from those calculated from maiden fish only. There is, therefore, no reason to believe that the results are not fairly reliable.

A. River Life.

The calculated lengths of fish in the different smolt classes are given in Table 23, and a graphical illustration is given in Fig. 3. It is obvious that the fastest growing fish migrated first, resembling in this respect the salmon of most rivers investigated to date. In the spring prior to migration a big proportion of the fish showed rings on the scales corresponding to freshwater

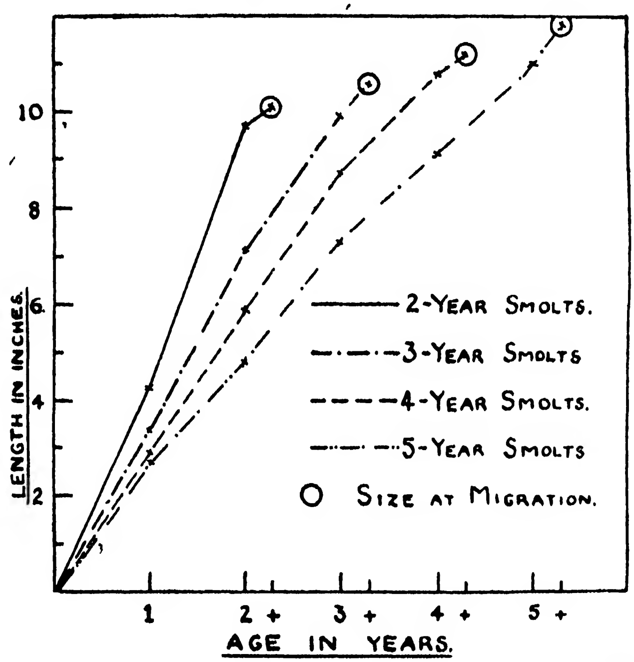

FIG. 3.—Growth curves of the various smolt ages in fresh water for sea trout.

growth. The junction between the portions of the scales representing freshwater and sea growth, respectively, was fairly clearly marked. It was, therefore, possible to calculate the length of the smolt in most cases. In Table 23 the calculated smolt size has been given. Smolts of sea trout (mean length 10·4 inches) of this river are much larger than those of other rivers in Ireland which have been investigated to date. There is a rise in the mean length of the smolt with an increase in smolt age. In Table 24 the calculated lengths in the different groups are given.

B. Sea Life.

Only one fish spawning for the first time in the fourth post migration winter was noted, the other fish all having become mature at least one year earlier (i.e.

by the third post migration winter). In Table 25 the calculated lengths at the end of the first, second, and third post migration winters are given. The average increment made in the first and second years is approximately the same, namely, 3·2 inches and 3·3 inches, respectively.

Average Sizes.

The average sizes are given in Table 26, and in those fish which had spawned for the first time in their second or third post migration winters the average length increased after each return from the sea. Before spawning the average growth in the sea amounted to 3·2 inches per annum, but after spawning the average increase in length between successive spawnings amounted to about half that amount. This is due partly to the fact that the actual period spent feeding in the sea is very much less between spawnings than in the maiden condition.

Oldest and Heaviest Fish.

Two fish (0·5 per cent.) were in their eleventh year since being hatched, 4 (0·9 per cent.) in their tenth year, 31 (7 2 per cent.) in their ninth year and 63 (23·1 per cent.) in their eighth year. The heaviest fish, measuring 30·0 inches in length, weighed 10 lbs. It was, however, somewhat below average condition, even for fish of this river, and would have weighed, in average condition, at least one pound more.

Discussion of Results.

In 1931 the late G. H. Nall published a report on sea trout of the Waterville River, based on 130 sets of scales and data, and his results were very similar to those described herein. The only differences worthy of note were the reduced condition co-efficients for 1941, and reduced percentage of fish in 1941 which had spawned for the first time in their first post migration winter, such differences being well within the range which might be expected from year to year.

The majority of the fish composing the spring runs of sea trout into the Waterville River are mature fish which have spawned in the previous winter and returned to fresh water after a short period feeding in the sea. Considering the very short period which they have spent in the sea in recovering from spawning, new sea growth was quite good, although after the first spawning growth fell off rapidly. Nall (1931) asks the question "why this overwhelming impulse towards a premature return involving a curtailment of growth." As Nall explains, it cannot be because they have a long journey to make, as the Waterville River and its tributaries are short, and the trout have only a short distance to go to the spawning grounds. Competent local observers believe that the sea trout entering the river in the spring are making their way into fresh water, and not merely running up and down with the tides. Indeed at present there is no real tidal portion of the river, as it enters the sea through a storm boulder beach, the pool just inside the mouth being unaffected by the

majority of tides. It is worthy of note that similar spring runs of mature fish are found in the Scottish Ailort which, like the Waterville River, is a short river with the spawning grounds comparatively near to the sea.

The growth rate in the sea, taking all classes of fish together, is higher than that found in most districts on the west coast of Scotland (Nall, 1930), whilst the growth rate in fresh water appears to be higher than in most other rivers investigated. It is possible that the late-running sea trout, which were not sampled, might give somewhat different results from those obtained by consideration of the spring runs. An attempt has been made this year to collect

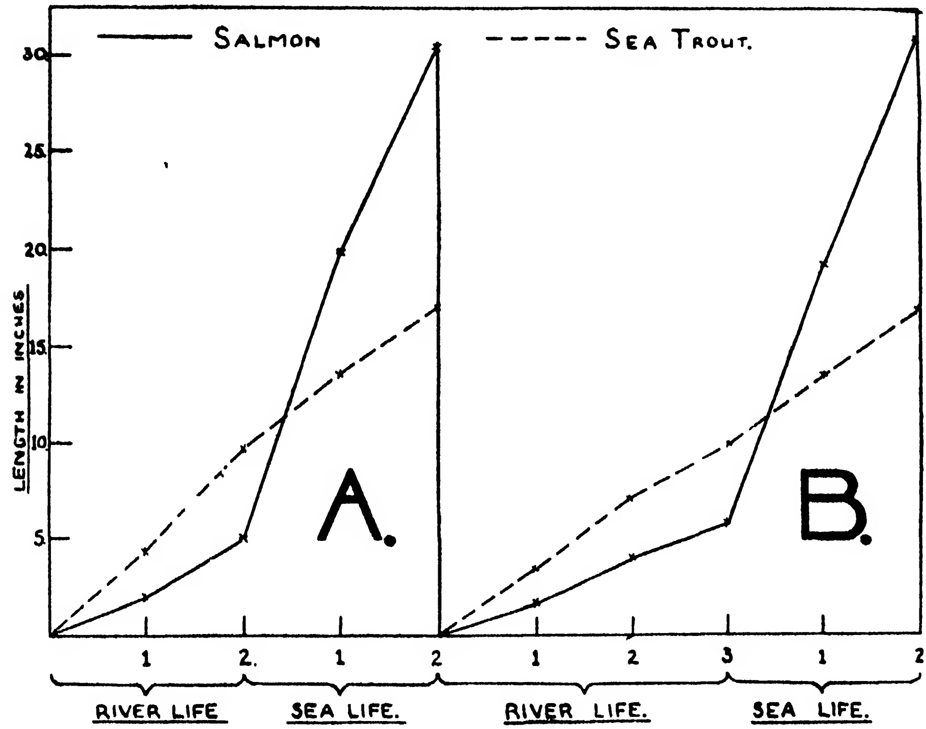

FIG. 4.—Growth curves of salmon and sea trout.
A. Two-year smolt class.
B. Three-year smolt class.

sufficient material to settle the matter, and to obtain a fairly accurate impression of the stocks of sea trout of this river as a whole. It was considered, however, that the results to date were of sufficient interest to include in this report.

A COMPARISON OF THE GROWTH OF SALMON AND SEA TROUT OF THIS RIVER.

The growth rates of salmon and sea trout of this river have been calculated from Tables 14 and 24 and are given in Table 27. A graphical illustration of these growth curves is given in Fig. 4. The salmon grow more slowly in fresh water than the sea trout, and, although the sea trout start their marine life

with a superiority of about four inches over the salmon, at the end of the first sea winter the salmon are approximately six inches longer than the sea trout. In the second sea year the gap between the two species becomes wider, so that the salmon are 13–14 inches longer than the sea trout.

Résumé.

(1) A description of the Waterville River and its catchment area is given and it is pointed out that the rocks of the area, belonging to the Old Red Sandstone, contain little or no lime. The waters of this system are either slightly acid or neutral. (Fig. 1 and Table 1.)

(2) Material consisting of sets of scales and data from salmon and sea trout was obtained from the net and weir fisheries on the Waterville River mainly during the year 1941.

(3) *Age and growth of the salmon.*—The bulk (86 0 per cent.) of the fish migrated as two-year smolts (Table 3). Small spring fish (2 winters) and grilse (1 + winters) formed 88·3 per cent. of the total catch (Table 4). The average condition co-efficient was 1 08, spring fish being superior in this respect to summer fish (Table 9). Average sizes in each age group have been given (Table 10). By means of scale measurements the length at end of each year of life in the maiden fish was calculated. In fresh water the fastest growing smolts migrated first (Table 11 and Fig. 2A). Some scales showed a large amount of growth in the spring prior to migration as a smolt. This type of growth was denoted by the term type B smolt growth, whilst, where there was little or no growth in fresh water in the spring prior to migration as a smolt, the growth was termed type A growth. Type A smolts were on the average longer at the end of each year of life in fresh water than type B smolts of the same smolt class (Table 13 and Fig. 2B). An explanation of this phenomenon was given. In the sea the grilse appear to be longer at the end of the first sea winter than any other age group (Table 14). The correlation of the lengths at the end of different years has been given (Tables 15, 16, and 17).

(4) *Age and growth of the sea trout.*—The bulk of the fish migrated as two- and three-year smolts in about equal proportions, a small proportion of four- and five-year smolts being noted (Table 20). Most of the fish were mature fish having entered the river and spawned previously (Table 19). Three-quarters of these fish had spawned for the first time as fish in their second post migration winter, and bore on their scales from one to five spawning marks. In general the fish of this river were of a slim type, the mean condition co-efficient being 0 9 on the Scottish Scale (Table 22). From the length at capture and measurements of the scales, the lengths at the end of the different years of life were calculated (Tables 23 and 24). Smolts of sea trout in this river are much longer than those of any other Irish river investigated. Before spawning the growth rate in the sea amounts to about 3·2 inches per annum, but after maturity this decreases to about half that amount. The average sizes have been given (Table 26). The oldest fish were in their eleventh year, and the heaviest fish measured 30 inches in length and weighed 10 lb.

(5) *Growth of salmon and sea trout compared.*—In fresh water the sea trout had a faster growth rate than the salmon in the two- and three-year smolt classes. Although the sea trout start marine life with an advantage of about four inches over the salmon, at the end of the first sea winter the salmon are approximately six inches longer than the sea trout. The advantage is maintained in the second sea year (Table 27 and Fig. 4).

The collection of the bulk of the material used in this investigation was made under the guidance of one of us (T. S. B.) who also prepared the scales for microscopical examination. The reading of the scales and subsequent working out of the results were carried out in the laboratory of the Fisheries Branch of the Department of Agriculture, Dublin, by the other author.

We wish to take this opportunity to express our thanks to Mr. J. W Butler, proprietor of the Waterville Fishery, for collecting most of the earlier material and for permission to make the collections during 1941. We are also indebted to Mr. Jeremiah Riordan, of Waterville, who made the necessary collections during 1941.

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APPENDIX (Tables 3 to 17 inclusive refer to salmon, and Tables 18 to 26 inclusive to sea trout).

Table 1. Analyses of waters of Waterville, Cummeragh, Coppal and Finglas Rivers and Lough Currane.

	Waterville River near Salmon Weir	Cummeragh River near Dromod	Coppal River (between Coppal Lough and Lough Currane).	Finglas River	Lough Currane near Church Island
Dissolved Solids					
Total	31.0	33.0	41.0	75.0	37.0
Fixed	2.9	28.0	25.0	61.0	30.0
Volatile	2.0	5.0	16.0	14.0	7.0
Chlorine (Cl)	18.0	10.0	16.0	25.0	19.0
Silica (SiO_2)	1.0	1.0	1.0	8.0	1.0
Iron (Fe_2O_3)	Nil	Nil	Nil	0.1	Nil
Calcium (CaO)	3.0	2.6	2.6	4.4	2.6
Magnesia (MgO)	Trace	Trace	Trace	2.0	Trace
Sodium (Na_2O)	12.2	12.5	12.2	27.5	12.6
Potassium (K_2O)	1.3	1.2	1.3	Trace	1.4
pH	6.3	6.6	6.5	6.3	6.3
Date of Sampling	23/8/1942	21/8/1942	22/8/1942	28/10/1941	23/3/1942

Table 2. Average Weight in lb. of salmon and sea trout taken in the Waterville Fishery in 1941

Month	Salmon		Sea Trout
January 1st-15th	11.9	11.9	
January 16th-31st	12.0		-
February	11.9		-
March	11.8		4.7
April	11.6		3.6
May	10.5		[illegible].4
June	6.9		3.2
July	6.8		3.0
Total	9.9		3.5

Table 3. Percentage of each smolt age in each age group.

Smolt Ages	Age Group (Winters)					Total Maiden Fish
	1+	2	2+	3	With S.M.s	
1	3.2	6.2	5.0	-	13.6	5.1
2	86.6	85.2	95.0	92.0	82.0	86.0
3	10.2	8.6	-	8.0	4.4	8.9
Total	100.0	100.0	100.0	100.0	100.0	100.0

Table 4. Percentage of each age group in the catch of each month.

Month	Age Group (Winters)					Total
	1+	2	2+	3	With S.M.s	
January	-	87	-	4	9	100
February	-	94	-	1	5	100
March	-	95	-	2	3	100
April	-	82	8	4	6	100
May	21	58	13	2	6	100
June	83	8	6	-	3	100
July	83	2	2	-	13	100
Total	32.9	55.4	2.5	2.0	7.2	100

Table 5. Percentages of the total catch of each age group in each month.

Month	Age Group (Winters)					Total
	1+	2	2+	3	With S.M.s	
January	-	28	-	49	24	18.2
February	-	31	-	11	14	18.1
March	-	25	-	22	6	14.8
April	-	7	15	12	4	4.8
May	4	6	29	6	5	5.6
June	42	2	42	-	8	16.9
July	54	1	14	-	39	21.6
Total	100	100	100	100	100	100.0

Table 6. "Absence habit" of previously spawned fish.

Absence Habit	Age at first spawning		Total
	1+ winters	2 winters	
Short (Less than one year)	11	-	11
Long (A full year)	-	32	32
Very long (More than a full year)	1	-	1
Total	12	32	44

Table 7. Showing divided migration and return. The table gives the years in which the fish examined were hatched as percentages of the total for the year.

Returned in 1941 as	Hatched in the year						Total
	1934	1935	1936	1937	1938	1939	
Grilse (1+ winters)	-	-	-	3.1	28.7	1.1	32.9
Small spring fish (2 winters)	-	-	4.7	47.2	3.5	-	55.4
Small summer fish (2+ winters)	-	-	0.2	2.3	-	-	2.5
Large spring fish (3 winters)	-	0.2	1.8	-	-	-	2.0
x Previously spawned fish (With S.M.s)	0.3	4.1	1.0	1.8	-	-	7.2
Total	0.3	4.3	7.7	54.4	32.2	1.1	100.0

x Approximate Values

Table 8. The estimated size distribution of salmon as percentages of the catch in 1941.

Class Interval in inches xx	Age Group (Winters)					Total
	1+	2	2+	3	With S.M.s	
22	5.46	-	-	-	-	5.46
24	10.91	-	-	-	-	10.91
26	13.20	2.07	0.13	-	0.17	15.57
28	3.12	14.10	0.25	-	0.86	18.33
30	0.21	22.68	0.88	-	1.20	24.97
32	-	13.36	0.62	-	1.20	15.18
34	-	[illegible].21	0.37	0.46	1.71	4.75
36	-	0.98	0.25	0.31	1.20	2.74
38	-	-	-	0.77	0.69	1.46
40	-	-	-	0.31	0.17	0.48
42	-	-	-	0.15	-	0.15
Total	32.9	55.4	2.5	2.0	7.2	100.0

xx Class interval 22 etc. includes all fish between 21.95 and 23.95 inches, etc.

APPENDIX—*continued.*

Table 9. Mean Condition Coefficients of the various age groups.

Age Group	Mean Condition Coefficient	Number Examined
1+ Winters	1.04	38
2 Winters	1.10	240
2+ Winters	1.00	5
3 Winters	1.13	8
With S.M.s	1.13	20
Total	1.08	311
All spring fish	1.10	267
All summer fish	1.05	44

Table 10. Mean lengths and estimated mean weights in the various age groups.

Age Group	Mean length in inches.	Estimated mean weight in lb.
1+ Winters	25.4	5.9
2 Winters	30.9	11.7
2+ Winters	31.8	11.6
3 Winters	38.0	22.7
With S.M.s	34.5	16.7

Table 11. Calculated lengths in inches in the various smolt classes in fresh water.

Smolt Age	Number Examined	Mean length at end of			Mean Smolt Length
		1st year	2nd year	3rd year	
1	33	3.2	-	-	5.2
2	553	2.0	5.0	-	5.7
3	56	1.7	4.0	5.7	6.0

Mean length of all smolts = 5.7 inches.

Table 12. Distribution of the two smolt types in the various smolt classes as percentages of the total catch of maiden fish.

Smolt Age	Type A smolts	Type B smolts	Total
1	-	5.1	5.1
2	13.4	72.6	86.0
3	5.0	3.9	8.9
Total	18.4	81.6	100.0

Table 13. Calculated mean lengths in inches in Type A and Type B smolts of the various smolt classes.

Smolt Age	Type A smolts					Type B smolts				
	Number Examined	Length at end of			Mean Smolt Length	Number Examined	Length at end of			Mean Smolt Length
		1st winter	2nd winter	3rd winter			1st winter	2nd winter	3rd winter	
1	-	-	-	-	-	33	3.2	-	-	5.2
2	70	2.3	6.2	-	6.2	483	1.9	4.8	-	5.6
3	32	1.7	4.1	5.8	5.8	24	1.6	3.8	5.5	6.3

Table 14. Calculated mean lengths in inches at end of each winter of River and Sea Life.

Age Group	Number Examined	River Life				Sea Life			
		Mean length at end of			Mean Smolt Length	Mean length at end of			Mean Length at Capture.
		1st winter	2nd winter	3rd winter		1st winter	2nd winter	3rd winter	
1.1+	5	3.3	-	-	5.3	20.5	-	-	26.8
2.1+	135	2.1	5.0	-	5.6	20.2	-	-	25.2
3.1+	16	1.7	4.0	5.8	6.2	20.1	-	-	25.1
1.2	27	3.2	-	-	4.9	19.0	30.9	-	30.9
2.2	389	2.0	5.0	-	5.5	19.4	30.7	-	30.7
3.2	39	1.7	4.0	5.7	5.9	19.3	31.0	-	31.0
1.2+	1	3.3	-	-	5.8	19.5	31.7	-	33.0
2.2+	18	1.8	4.8	-	5.7	18.5	29.7	-	31.8
2.3	11	2.0	5.2	-	5.8	19.7	31.2	38.2	38.2
3.3	1	1.0	3.0	4.4	5.2	19.5	31.4	38.0	38.0

Table 15. Correlation between lengths at end of first and second winter in the two-year smolt class.

Length at end of 1st winter	Number examined	Mean length at end of 2nd winter	Mean Increment made in 2nd year
0.95-1.45 ins.	74	3.9 inches	2.7 inches
1.45-1.95 ins.	188	4.5 inches	2.8 inches
1.95-2.45 ins.	182	5.3 inches	3.1 inches
2.45-2.95 ins.	85	5.8 inches	3.1 inches
2.95-3.45 ins.	21	6.6 inches	3.4 inches
3.45-3.95 ins.	2	7.7 inches	4.0 inches

Table 16. Correlation between smolt length and length at end of first sea winter in the grilse and small spring fish.

Smolt length	Grilse		Small spring fish	
	Number examined	Mean length at end of first sea winter	Number examined	Mean length at end of first sea winter
Less than 4.95 ins.	27	18.9 ins.	87	18.2 ins.
4.95-5.95 ins.	65	20.0 ins.	191	19.2 ins.
More than 5.95 ins.	64	21.3 ins.	171	20.8 ins.

Table 17. Correlation between lengths at end of first and second sea winters in the small spring fish (2 winters age group).

Length at end of first sea winter	No. examined	Mean length at end of second sea winter	Mean increment in second sea winter
Less than 18.95 ins.	177	29.0 ins.	11.1 ins.
18.95 - 19.95	131	30.9 ins.	11.4 ins.
Above 19.95 ins.	146	32.4 ins.	11.2 ins.

Table 18. Monthly catches, as percentages of the total catch for the year 1941.

Month	Percentage Monthly Catch
February / March	7.2%
April	27.6%
May	31.6%
June	30.5%
July	3.1%
Total	100.0%

Table 19. Monthly proportion of maiden and previously spawned fish.

Month	Maiden Fish	Previously spawned fish
February / March	10%	90%
April	7%	93%
May	17%	83%
June	19%	81%
July	62%	38%
Total	15.7%	84.3%

Appendix—*continued.*

Table 20. Proportion of the different smolt ages.

	Smolt age				Total
	Two-year	Three-year	Four-year	Five-year	
Number of Individuals Percentage of Total	201 46.5%	198 45.7%	33 7.6%	1 0.2%	433 100.0%

Table 21. Age at first spawning in the different smolt classes.

Age at first spawning	Smolt Age								Total	
	2		3		4		5			
	No.	%	No.	%	No.	%	No.	%	No.	%
First Post-Migration year	6	4	13	7	8	29	1	100	28	7
Second Post-Migration year	134	77	129	74	18	64	-	-	281	75
Third Post-migration year	32	19	32	19	2	7	-	-	66	18
Total	172	100	174	100	28	100	1	100	375	100

Table 22. Mean monthly condition coefficients.

Month	Number examined	Mean Condition Coefficient
February March	31	0.89
April	115	0.92
May	60	0.85
June	32	0.92
July	4	0.88
Total	242	0.90

Table 23. Calculated mean lengths in inches in fresh water.

Smolt Age	Number	Mean length at end of					Mean Smolt length
		1st Winter	2nd Winter	3rd Winter	4th Winter	5th Winter	
2	199	4.3	9.7	-	-	-	10.1
3	189	3.4	7.1	9.9	-	-	10.6
4	32	2.9	5.9	8.7	10.8	-	11.2
5	1	2.7	4.8	7.3	9.1	11.0	11.8

Table 24. Calculated mean lengths in inches at the end of each year of life in the river and sea.

Group	Number Examined	River Life						Sea Life		
		Mean length at end of					Mean Smolt Size	Mean length at end of		
		1st winter	2nd winter	3rd winter	4th winter	5th winter		1st winter	2nd winter	3rd winter
2+	7	4.5	9.3	-	-	-	9.9	-	-	-
3+	11	3.7	8.1	10.9	-	-	11.4	-	-	-
4+	7	3.1	6.2	9.1	11.4	-	11.9	-	-	-
5+	1	2.7	4.8	7.3	9.1	11.0	11.8	-	-	-
2.1+	145	4.3	9.8	-	-	-	10.[illegible]	13.7	-	-
3.1+	134	3.4	7.2	9.8	-	-	10.6	13.4	-	-
4.1+	20	2.9	5.8	8.7	10.8	-	11.0	14.3	-	-
2.2+	46	4.3	9.3	-	-	-	10.2	13.3	16.9	-
3.2+	44	3.2	6.6	9.7	-	-	10.4	13.7	16.9	-
4.2+	5	2.7	5.9	8.4	10.4	-	11.0	14.7	17.5	-
2.3+	1	3.1	9.2	-	-	-	10.5	1[illegible].0	15.	18.1

Table 26. Average sizes in the different age groups. Mean weight in last column being calculated on basis of k = 0.9.

Age Group	Number examined	Length in inches			Mean Weight
		Minimum	Maximum	Mean	
.+	1	-	-	12.0	0.7
.+S.M.+	3	-	-	18.7	2.5
.+2S.M.+	8	20.0	23.5	21.7	3.8
.+3S.M.+	8	20.0	25.0	21.7	3.8
.+4S.M.+	5	20.9	24.5	22.3	4.3
.+5S.M.+	3	20.9	23.2	22.1	4.1
.1+	27	13.5	20.0	17.1	1.9
.1+S.M.+	76	14.5	24.5	19.1	2.7
.1+2S.M.+	97	18.5	25.0	21.5	3.8
.1+3S.M.+	77	18.2	25.5	22.7	4.5
.1+4S.M.+	23	20.9	25.0	23.4	4.9
.1+5S.M.+	6	23.1	26.0	24.6	5.7
.1+S.M.I+	1	-	-	24.0	5.3
.2+	30	17.5	24.0	20.6	3.4
.2+S.M.+	38	19.0	25.0	22.2	4.3
.2+2S.M.+	13	21.0	25.5	22.9	4.6
.2+3S.M.+	12	22.0	30.0	24.4	5.6
.2+5S.M.+	4	24.0	27.0	25.3	6.2
.3+	1	-	-	19.5	2.8
Total	433	12.0	30.0	-	-

Table 25. Calculated mean lengths in inches in the sea.

Age Group	Number examined	Mean length at end of		
		1st winter	2nd winter	3rd winter
2nd Post Migration Summer	299	13.6	-	-
3rd Post Migration Summer	95	13.6	16.9	-
4th Post Migration Summer	1	13.0	15.2	18.1
Total	395	13.6	16.9	18.1

Table 27. Growth rates of salmon and sea trout in the two-and three-year smolts.

Type of Fish	Calculated mean lengths in inches				
	River			Sea	
	1st year	2nd year	3rd year	1st year	2nd year
Two-year Smolt Class					
Salmon Sea Trout	2.0 4.3	5.0 9.7	- -	19.3 13.6	30.5 16.9
Three-year Smolt Class					
Salmon Sea Trout	1.7 3.4	4.0 7.1	5.7 9.8	19.5 13.5	31.0 16.9

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ASCORBIC ACID.

PART I: DETECTION AND ESTIMATION.

BY W. R. FEARON AND EINHART KAWERAU.

Price One Shilling.

No. 10.

ASCORBIC ACID.

Part I: Detection and Estimation.

By W. R. FEARON and EINHART KAWERAU.

[Read June 23, 1942. Published separately April 16, 1943.]

The present inadequate supply of imported citrus fruits and the scarcity of many home-grown fruits have led to the belief that the Irish national dietary may be exposed to the dangers of a deficiency in vitamin C. For this reason a survey has been undertaken, with the assistance of a grant from the Medical Research Council of Eire, the object being to explore and assess available sources of supply. In addition, a study has been made of the stability of the vitamin, especially with regard to the effects of storage, cooking, and industrial treatment. The first part of the work now submitted deals with the methods available for the detection and estimation of vitamin C, and describes new reactions for the detection of both the reduced and the oxidised form of the vitamin.

Vitamin C, as is generally known, can occur in three forms: (1) free ascorbic acid, (2) oxidised or dehydro-ascorbic acid, and (3) "bound" ascorbic acid, the nature of which is somewhat obscure. All three forms are biologically equivalent in that they are interchangeable in human nutrition, but the fact that they differ in their chemical properties introduces complications into the procedure for estimating the vitamin content of natural food materials. Thus, when dealing with plant or animal tissue, it is customary to extract with an acid solvent in order to liberate any "bound" ascorbic acid that may be present. The extract is then treated with hydrogen sulphide to reduce any dehydro-ascorbic acid present, and the resulting solution of ascorbic acid is then titrated by means of an appropriate indicator. Many errors may arise in this procedure.

The outstanding characteristic of ascorbic acid is its ability to act as a powerful reducing agent, or hydrogen-donor, even in strongly acid solutions. This property is revealed by the rapid reduction of various reagents used from time to time by various workers for the detection of the vitamin. These reagents are represented by: (1) Ag^{+} in acid solution (Szent-Györgyi, 1928), (2) Cu^{++} (Szent-Györgyi, 1928), (3) Au^{+++} (Emmerie, 1935), (4) Fe^{+++} (Tauber and Kleiner, 1935; McFarlane, 1936), (5) Hg^{++} (Pittarelli, 1936), (6) V_2O_5 (Freses, 1938), (7) H_2SeO_3 (Emmerie, 1935), (8) Ceric acid (Süllman, 1938), (9) Tungstic acid (Medes, 1935; Fujita *et al.*, 1935, 1938; Shinohara

and Padis, 1936), (10) Molybdic acid (v. Euler and Burström, 1936), (11) Iodine (Szent-Györgyi, 1927, 1928, (12) Dibromophenol-indophenol (Tillmans *et al.*, 1932), (13) Dichlorophenol-indophenol (Tillmans *et al.*, 1932), (14) Methylene blue in presence of light (Martini and Bonsignore, 1934; Lund and Lieck, 1936; Gál, 1936), (15) Cacothelin (Rosenthaler, 1938), (16) Dichlorobenzene-indophenol (Morell, 1941). To this list we now add (17) *o*-dinitrobenzene, which we find is rapidly reduced in alkaline solution by the vitamin, yielding a violet pigment.

The *o*-Dinitrobenzene Test for Ascorbic Acid.

To 5 ml. of the vitamin solution add 2–4 drops of a saturated aqueous solution of *o*-dinitrobenzene, followed by 20 drops of 20 per cent. sodium hydroxide. If more than 1·5 mg. of ascorbic acid be present, a violet colour develops in a few seconds, and reaches maximum intensity in about 10 minutes at ordinary temperature. In weaker solutions colour development is proportionately slower, the rate depending on the temperature and degree of alkalinity. Under the above conditions, the reaction is not given by dehydro-ascorbic acid, glutathione, cysteine, creatinine, or uric acid. The reducing sugars eventually give the reaction, but only when in much higher concentrations and after remaining for at least half-an-hour in contact with the reagent. If the mixture is heated it loses its selectivity, and many reducing agents give the reaction. The violet pigment has been isolated and identified as the salt of the nitroxylic acid formed by simple reduction of the dinitrobenzene (Fearon and Kawerau, 1943).

Dehydro-ascorbic Acid.

Dehydro-ascorbic acid, the oxidised form of vitamin C, is usually detected indirectly by conversion to ascorbic acid. Szent-Györgyi (1928) was the first to show that this could be quantitatively effected by hydrogen sulphide, and by this means dehydro-ascorbic acid has been shown to occur in many plant and animal tissues (Bessey and King, 1933; Van Eeklen *et al.*, 1934; Bacharach *et al.*, 1934; Gabbe, 1934). Other methods of reduction have been advocated, such as stannous chloride (Roe, 1936). Our own observations indicate that neither magnesium amalgam nor aluminium amalgam is effective, and Barron *et al.* report similar negative results from the use of nascent hydrogen in presence of palladium (1935). A serious source of error following the use of hydrogen sulphide has been observed by King (1941), who has shown that many naturally-occurring aldehydes, ketones, and quinones are converted by hydrogen sulphide into compounds that reduce the titration indicators in a manner similar to ascorbic acid.

The actual amount of dehydro-ascorbic acid originally present in a tissue is also somewhat uncertain, since catalytic oxidation of the vitamin is liable to occur at some stage in the analytical treatment. This is usually due to

copper either in ionic form or associated with a protein, such as the so-called "ascorbic oxidase" enzyme. Barron *et al.* (1935) have shown that 46 micrograms of copper per litre are capable of effecting a 50 per cent. oxidation of the ascorbic acid present, in six hours at pH 1·07.

Copper may be introduced into the system in three ways: (1) by the tissue itself; (2) by the distilled water used in making the extracts and preparing the reagents; and (3) by the actual reagents. By the use of glass-distilled water and specially purified reagents, copper-contamination can be lessened, but the influence of copper derived from the tissue can only be overcome by the use of a reactant that will depress the ionisation of the metal. Metaphosphoric acid has been found to be effective for this purpose (Fujita and Iwatake, 1935), and is widely employed. Copper immobilisation also inhibits the oxidation of ascorbic acid by enzymes, since it forms part of the prosthetic group of the oxidase system (Kubowitz, 1937, 1938; Lovett-Janison and Nelson, 1940; McCarthy *et al.*, 1939; Meiklejohn and Stewart, 1941).

Only one direct test for dehydro-ascorbic acid appears to have attracted attention up to the present. On treatment with 2: 4-dinitro-phenylhydrazine an osazone is formed, which can be separated and estimated gravimetrically or by titration with titanium chloride, or colorimetrically by the red colour it yields when dissolved in concentrated sulphuric acid (Roe and Kuether, 1942).

THE ACID TEST FOR DEHYDRO-ASCORBIC ACID.

When a solution of dehydro-ascorbic acid is buffered to the region of pH 4 and gently boiled, we have observed that it develops a stable grass-green colour. The test is not given by ascorbic acid, or by any of the familiar biological acids, sugars, proteins, and related compounds. The test may be demonstrated by carefully oxidising 5 ml. of dilute (0 01–0 001 per cent.) ascorbic acid by addition of one per cent. iodine solution. Any excess of the halogen must be removed by addition of more ascorbic acid or a particle of thiourea. The mixture, before or after oxidation, is set to pH 3–4 by addition of about 5 g. of sodium acetate and 10 drops of glacial acetic acid. On boiling for a couple of minutes the characteristic green colour appears, and may be diluted with water for the purpose of colorimetric comparison. The possible mechanism of the test is discussed elsewhere (Fearon and Kawerau, 1943).

"BOUND" ASCORBIC ACID.

Fujita and Ebihara (1939) conclude that some at least of the ascorbic acid present in tissues occurs as a "bound" form, that can be released by metaphosphoric acid extraction or by boiling, provided that the destructive effects of copper and oxidising enzymes are properly controlled. This conclusion appears to have been misapprehended by Harris and Olliver (1942) in their recent review. The increased yield of indophenol-reducing material obtained by

boiling has also been noted by Mack and Tressler (1937), and by Borsook *et al.* (1937), and has been attributed to formation of diketo-gulonic acid, a degradation product of ascorbic acid that is more strongly reducing than the parent compound. Harris and Olliver report their complete failure to find evidence for the existence of "bound" ascorbic acid in vegetable material.

"Bound" ascorbic acid has also been reported to occur in urine (Scarborough and Stewart, 1937; Guha and Sen-Gupta, 1938, 1940). Holts and Walter (1940) postulate a protein-bound ascorbate, which can be released by acid or enzyme (pepsin or papain) hydrolysis. We have some indications that, in the potato, dehydro-ascorbic acid can exist in a complex form less susceptible to oxidative destruction than either of the free forms of the vitamin.

Estimation of Ascorbic Acid.

Application of the new tests to biological material leads us to agree with the conclusions of Harris and Olliver (1942), Stone (1937), and Bessey (1938), all of whom maintain that most of the vitamin C of the dietary, in as far as it is derived from fresh foodstuffs, consists of free ascorbic acid in the reduced state. The tests that depend on the reducing potency of the tissue extracts and products are hence of primary importance, and we have examined their quantitative application to the assay of the vitamin.

1. *Silver*: Although the metallic ion is very readily reduced by traces of ascorbic acid, it is not possible to titrate either reactant by means of the other, since there is no sharp end-point obtainable. The reduced silver appears in a colloidal form, and obscures the titration when carried out in presence of adsorption indicators, such as tartrazol, even when back-titrated with standard chloride solutions at varying degrees of acidity.

2. *Copper*: Ascorbic acid at room temperature rapidly reduces the alkaline sugar reagents, such as those of Fehling or Benedict. But, as in the silver solutions, the end-point is not sharp, and there is a considerable lag in the final stage of the reaction.

3. *Tungstic Acid and Molybdic Acid*: In a series of papers, Fujita and his colleagues (1935, 1937, 1938) discuss the reduction of phosphotungstic and phosphomolybdic acids by ascorbic acid, and conclude that the reagents employed by earlier workers (Bezssonoff, 1935; Euler and Burström, 1936) are not selective enough to be of value in the estimation of small quantities of the vitamin. They also found that Folin's molybdic reagent, at first thought to be suitable, has the defect that the blue pigment it yields with the vitamin fades, even at freezing-point, and thus makes colorimetric work untrustworthy. Since alkali is used in the development of these pigments the method has the further disadvantage of being inapplicable to vegetable extracts rich in flavones, which give intense yellow colours on addition of alkali.

Fujita and Ebihara (1938) overcome this difficulty by using the Folin reagent at pH 3, and destroying non-ascorbic reducing substances by means of iodoacetic acid. The method, however, requires the use of the photometric colorimeter, and specially prepared filters.

4. ***Methylene Blue***: This dye has been advocated as more selective for ascorbic titration than the indophenols (Martini and Bonsignore, 1934; Wachholder and Podesta, 1936), since it has a lower redox potential, and therefore is less affected by non-ascorbic reducing substances, especially those of the thiol class. Gál (1936) and Lund and Trier (1939) have devised apparently satisfactory methods based on the bleaching of methylene blue, but Armentanó (1940), after a critical survey of the subject, concludes that the titration is so sensitive to copper and iron ions that only a rigid control of the pH and other conditions can exclude errors due to re-oxidation of the leuco-form of dye by atmospheric oxygen.

The methods finally adopted in the present work were: (1) ferric titration for ascorbic acid in phosphate-free solutions, (2) iodine titration for ascorbic acid in biological concentration of more than 10 mg. per 100 ml., and (3) dichlorophenol-indophenol titration for ascorbic acid in concentrations less than 10 mg. per 100 ml.

ESTIMATION OF ASCORBIC ACID BY FERRIC TITRATION.

This method is based on the quantitative reduction of Fe^{+++} to Fe^{++} by ascorbic acid in acid solution, thiocyanate being used as an internal indicator. Its application to biological material is limited by the fact that phosphates interfere by precipitating Fe^{+++}; apart from this, the method is suitable for studying the course and rate of ascorbic oxidation in presence of various catalysts.

Reagents: 1. Standard Iron solution containing 0·1 mg. Fe^{+++} per litre, prepared by dissolving 0·702 g. of analytically pure ferrous ammonium sulphate in 100 ml. of distilled water. Five ml. of sulphuric acid diluted 1 in 5 are added. The iron is then oxidised by exact addition of 0·2 per cent. potassium permanganate until a faint pink colour persists. The solution is then diluted to 1 litre and well mixed. 2. Indicator, 1 per cent. potassium thiocyanate.

Method: An appropriate quantity of solution for analysis, 1 to 10 ml., is acidified with a few drops of glacial acetic acid. One ml. of indicator is added, and the mixture titrated with the standard iron solution from a micro-burette until a definite pink shade persists. At 20° C. the speed of titration is rapid almost up to the end-point. On the assumption that 2 atoms of iron are reduced by one molecule of ascorbic acid, 1 ml. of standard iron solution (= 0·1 mg. Fe^{+++}) is equivalent to 0·157 mg. of ascorbic acid. This value is five times less than the corresponding ascorbic equivalent of the N/100 iodine used in the alternative method, and thus the iron titration is better suited for estimation of small amounts of vitamin C in absence of phosphate interference.

ESTIMATION OF ASCORBIC ACID BY IODINE TITRATION.

Working with suitable material, iodine has been shown to yield good results in ascorbic acid estimations (Szent-Györgyi, 1928; Ballentine, 1941). The reagent (N/100 Iodine) is easily standardised against thiosulphate, and is

much superior in keeping qualities to dichlorophenol-indophenol. Its chief disadvantage is due to its reduction by thiol compounds, notably glutathione, for the estimation of which it has frequently been used, and these compounds should be absent from the extracts being assayed for vitamin C. The -SH group in biological thiols can be rendered inactive by addition of heavy metals, such as Hg^{++}, but these ions are liable to catalyse the oxidation of ascorbic acid, or combine with it to form salts. Iodine also tends to be adsorbed by proteins or polysaccharides present in the extracts, and thus give unduly high titration values. Using a 1 per cent. solution of soluble starch as indicator, we have observed that 2 drops added to 1 ml. of titration substrate are capable of adsorbing iodine to the equivalent of 0·02 mg. of ascorbic acid. This effect makes it difficult to titrate a colloidal solution such as milk, and the method is useful chiefly for the estimation of ascorbic acid in simple solutions and colourless fruit juices. Where the extracts are pigmented, the bleaching of the iodine may not be observable, and the starch indicator is obscured. To overcome these limitations, we have used xylene or benzene as a partition solvent, whereby a trace of free iodine in the completed titration can be detected. In practice, about 0 5 ml. of xylene is added to the solution being titrated. After each addition of iodine, the tube is shaken gently, and the xylene allowed to separate out. The end-point is shown by the appearance of a pink colour in the xylene layer. The method is sensitive, and will reveal one drop of N/1000 iodine added to 10 ml. of water. Natural fruit pigments and the dyestuffs commonly employed to colour preserves are not extracted by xylene, so the method is widely applicable to analyses of commercial products. The utility range of the method extends down to ascorbic values of about 10 mg. per 100 ml.

Estimation of Ascorbic Acid by Dichlorophenol-Indophenol.

Dichlorophenol-indophenol is a highly sensitive titration reagent for ascorbic acid. The optimum conditions have been exhaustively worked out (Tillmans *et al.*, 1932; Birch *et al.*, 1933; Harris and Olliver, 1942), and we have nothing new to add. It is the most suitable method when dealing with ascorbic concentrations below 10 mg. per 100 ml., or whenever the iron or iodine methods are inapplicable.

Although the dye is bleached by glutathione and by cysteine, even in solutions as acid as pH 3, the rate of reduction by these compounds is much slower than reduction by ascorbic acid, and, by rapid titration, consistent values for the vitamin are obtainable with practice.

Unfortunately, the dye is very unstable in aqueous solution, and must be standardised every time it is used. This may be done by means of pure crystalline ascorbic acid, a reagent not readily obtainable, or by standardisation in bulk, using other reducing agents (Menaker and Guerrant, 1938). Ramsey and Colichman (1942) avoid the need for an accurately standardised dye by a double titration method, involving the use of iodine. We have endeavoured to

develop this method, and the results will be submitted in a later communication.

For the purpose of checking the strength of the solutions prepared from the tablets of dyestuff now on the market, we have made use of our observation that the colour of dichlorophenol-indophenol in acetic acid solution can be very closely matched by thymol blue in a glycerol solution at pH 2·0.

Where the solution being titrated is already pigmented, chloroform, as suggested by McHenry and Graham (1935), can be used to extract the dye, and thus reveal the colour change at the end-point. Chloroform, however, is a solvent for some natural pigments, and, when these are present, other more selective solvents, such as a mixture of xylene and amyl alcohol, may be used (Lanke, 1939).

ESTIMATION OF DEHYDRO-ASCORBIC ACID.

Preliminary work shows that it is possible to estimate dehydro-ascorbic acid in unpigmented extracts by means of the colour reaction obtained in acid solutions. The green product can be accurately matched in a colorimeter by a standard solution of uranium acetate or nitrate, and obeys the Beer-Lambert law over a sufficiently wide range of concentration to cover the requirements of ordinary biological analyses.

SUMMARY.

1. The available methods for the detection and estimation of ascorbic acid, dehydro-ascorbic acid, and "bound" ascorbic acid are classified and discussed.

2. A new colour test for ascorbic acid is described, depending on the reduction of *o*-dinitrobenzene in alkaline solution at room-temperature.

3. A new colour test for dehydro-ascorbic acid is described, depending on the formation of a green pigment when the vitamin is boiled in solution buffered to pH 3–5.

4. A method for the estimation of ascorbic acid by ferric titration, using thiocyanate as indicator, is described.

5. A method for the estimation of ascorbic acid by iodine titration, using xylene as a partition indicator, is described.

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ON AN EPIDEMIC OF *GIBBERELLA SAUBINETII* (Mont.) Sacc. ON WHEAT IN EIRE IN 1942.

By ROBERT McKAY, D.Sc.,
Lecturer in Plant Pathology, University College, Dublin.

Plates 4 and 5.

Price Three Shillings.

No. 11.

ON AN EPIDEMIC OF *GIBBERELLA SAUBINETII* (Mont.) Sacc. ON WHEAT IN EIRE IN 1942.

By ROBERT McKAY, D.Sc.,
Lecturer in Plant Pathology, University College, Dublin.

PLATES 4 AND 5.

[Read MARCH 23. Published separately MAY 28, 1943.]

INTRODUCTION.

IN Great Britain and Ireland the disease known as "Ear Blight" of cereals is one in which the spikes or panicles are attacked by a fungus that forms more or less a weft of mycelium on affected spikelets and interferes with the normal development of the grain. The term "Ear Blight," however, refers to the effects of a number of closely similar species of *Fusarium*. Most of these cannot be recognized specifically from a mere superficial examination of diseased heads or grain, and very often the actual species involved can only be identified after considerable cultural work in the laboratory. A search of the records kept during the past twenty years in this country shows that, in the case of "Ear Blight" of wheat, while *Fusarium* spp. (representing unidentified species) have occasionally been recorded, the species usually associated with this disease have been *F. avenaceum, F. herbarum,* and *F. culmorum.* To these three, a fourth, namely, *Fusarium graminearum* Schwabe (*Gibberella Saubinetii* (Mont.) Sacc.[1]), must now be added. As Lafferty found this organism on wheat grain in 1918 in Ireland, it probably figured amongst the unidentified species during the past twenty years, but in the absence of cultural work it escaped recognition.

In attacks due to the four organisms, *Fusarium avenaceum, F. herbarum, F. culmorum* and *F. graminearum* Schwabe, the symptoms of disease on wheat ears are all alike. Each of these organisms gives rise to a mycelium which varies from dull or faint pink to carmine in colour. Affected grains are often similarly coloured, either partially or wholly, the embryo end of the grain generally showing the greatest development of red pigment. Though *F. graminearum* (*G. Saubinetii*) is stated to be the most common cause of reddish discoloured wheat grains, according to Atanasoff (2) other fungi may

[1] According to Petch the correct name of the fungus causing "Scab" on cereals is *Gibberella Zeae* (Schwein.) Petch. For full discussion on this question of nomenclature see Petch, T.—Ann. Mycol., Berl., 34, pp. 256–260, 1936. In the absence of general acceptance of Petch's claim the present writer prefers to adhere to *Gibberella Saubinetii* (Mont.) Sacc., the name by which the fungus is most widely known.

occasionally be responsible for it, and reddish discoloured grains are not, therefore, indicative of any single organism. In *Fusarium avenaceum*, *F. herbarum*, and *F. culmorum* asexual fructifications alone occur, being produced both on diseased grain and in pure cultures, but *Fusarium graminearum* Schwabe is recognized as being simply the conidial stage of the fungus *Gibberella Saubinetii* (Mont.) Sacc. Hence, the production of typical perithecia containing the characteristic asci and ascospores is the diagnostic feature in this case.

While attacks of an orange-coloured fungus on wheat ears were first described in England in 1884, being reported as a new disease under the name *Fusisporium culmorum* W. Sm. (30), and other species of *Fusisporium* (*Fusarium*) were even then known to occur on various cereals, it was not until 1930 that the fungus *Gibberella Saubinetii* (Mont.) Sacc. was definitely recorded on British cereals. Bennett (4) isolated this fungus from discoloured wheat grains in 1928, and on incubation of some of the naturally infected grain early in the following year he obtained perithecia, a number of which eventually developed asci and ascospores. Bennett also obtained perithecial development on artificially inoculated wheat grains brought to maturity on sterile soil. The same worker afterwards isolated *Gibberella Saubinetii* from bases of barley, oats, wheat, rye, and *Triticum monococcum* plants, and showed that, though the fungus was widespread throughout England, the climatic conditions there were not favourable for perithecial formation (5). Finally, however, Bennett (6) reported the fortuitous occurrence of these fructifications on the ears of Little Joss wheat in 1932, this being the first record (and apparently up to the present time the only one) of perithecial development in England under natural conditions.

In addition to Bennett's papers (4), (5), (6) on *G. Saubinetii*, the only other reference to the occurrence of this fungus on cereals in Great Britain is that by Dennis and Foister (9), where it is listed as occurring on wheat in Scotland.

Bennett in one of his papers (5) refers to an unpublished record by Lafferty of *G. Saubinetii* on wheat in Co. Galway, Ireland, in 1918. From information supplied to the writer verbally by Dr. Lafferty, it now transpires that during a study of *Fusarium* species at that time, he isolated *F. graminearum* from discoloured wheat grains, and later obtained perithecia in pure cultures of the organism, thus settling the question of identification. This isolation of the fungus by Dr. Lafferty is the only previous authentic evidence of the presence of *Gibberella Saubinetii* in this country, but it has doubtless been endemic in many districts for a long time.

Though the number of publications in Great Britain and Ireland on *Gibberella Saubinetii* on cereals is small, the fungus has been intensively studied in other countries, particularly in America. The importance of this organism lies not only in the actual loss sustained by the grower through "Ear Blight," and to a less extent through "Seedling Blight," but also to the well-known toxic effect both of diseased grain and of the manufactured products.

The present paper is an account of an epidemic of *Gibberella Saubinetii* (mainly on wheat) in Eire in 1942, together with some details of actual losses incurred. The latter feature of the disease is one on which practically no information has hitherto been available in these islands.

The Occurrence of *Gibberella Saubinetii* on Wheat in Eire in 1942.

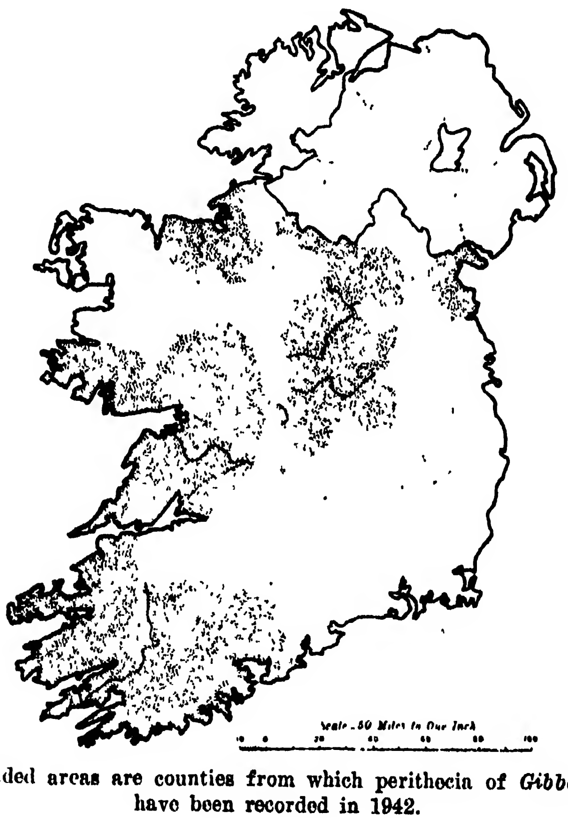
Fungus attacks on wheat ears, due to a species of *Fusarium*, were observed in several localities in Eire in 1942, the disease being present from the flowering time of the host onwards. As the season advanced, complaints from growers became numerous when it was seen that wheat ears were not filling out properly, and by harvest time it was obvious that serious losses had occurred in certain districts. A couple of examples from different counties will suffice to show what was taking place.

On 28th August, diseased wheat specimens, accompanied by a report, were received from Co. Kerry, the correspondent stating that "the crop from which the sample was taken was not worth cutting and that many crops of wheat in his district were similarly affected." The fungus attack on the wheat heads received was severe, and while certainty about the correct identification of the *Fusarium* species present was not then possible, later, on receiving further specimens from the same crop, the fungus was identified as *Fusarium graminearum* Schwabe, the conidial stage of *Gibberella Saubinetii*. This was afterwards confirmed when many heads of wheat with mature perithecia of the fungus were received from the correspondent. The second example was from the midlands. On the 23rd September, Mr. P. Conroy, Agricultural Instructor, Co. Westmeath, forwarded a few wheat ears for the identification of a fungus which was forming black incrustations on the spikelets. The black crusts on the wheat ears in this case proved to be composed largely of mature perithecia of *G. Saubinetii*. In answer to an enquiry regarding the prevalence of the disease on the crop, with a request for further specimens and information concerning the outbreak, Mr. Conroy kindly sent some sixty wheat heads with perithecia on them, and also supplied the following observations:—"The pink *Fusarium* stage of the fungus was prevalent throughout the crop, and black spotted heads with perithecia occurred to the extent of one ear to every two square yards over the whole area, which was slightly above two statute acres. The wheat ears did not turn down in the characteristic manner of a full crop. The workmen commented on the peculiar taste of the grain, the lightness of the sheaves, and the absence of grain-feel in the ears."

As other specimens with perithecia of *G. Saubinetii* and several samples of pink discoloured grain came to hand, the co-operation of the Department of Agriculture was sought in order to ascertain to what extent the disease was distributed throughout the country. Unfortunately, the Department was not approached until the season was well advanced, many of the wheat crops were

already harvested, and a considerable number had been threshed. The Department, however, immediately sent out a memorandum to all the Agricultural Instructors in the various counties of Eire, asking that any wheat (either in grain or in ear) bearing evidence of this disease might be forwarded for examination. In the case of grain already threshed, a sample of the chaff was requested, if available, and attention was drawn to the blackening of the glumes with an appearance similar to smut. Information was also requested regarding (*a*) variety of wheat affected, (*b*) seed dressing, if any, before sowing, and (*c*) particulars of previous cropping.

As a result of this memorandum, many samples of diseased wheat were received, some showing only the *Fusarium* stage, but others displaying the perfect fructifications. Specimens of wheat with mature perithecia of the fungus *Gibberella Saubinetii* have now been received from the following



Map of Eire, shaded areas are counties from which perithecia of *Gibberella Saubinetii* have been recorded in 1942.

counties:—Louth, Cavan, Sligo, Galway, Clare, Kerry, Cork, Westmeath, Longford, and Offaly. Therefore, the disease is widespread throughout Eire, as can be seen from a glance at the accompanying map.

As the criterion for the identification of the fungus was the presence of typical perithecia containing asci and ascospores, and the investigation was

started too late in the year to accomplish a systematic survey, it cannot be assumed that the remaining counties are necessarily free from the disease. Evidence on this point will be adduced later in this paper.

The varieties of wheat on which perithecia of *Gibberella Saubinetii* were found are the following:—

Queen Wilhelmina	Diamant.
Red Marvel.	Squarehead's Master.
April Red.	

The first, Q. Wilhelmina, made up to 54 per cent. of the diseased specimens, but as this variety is more widely grown than any of the others, it was only natural that more of it would be received. At the same time, perithecial development on this variety appeared to be more prolific than on any of the others mentioned, and Q. Wilhelmina may be more susceptible to the disease.

The following data show previous cropping and treatment of wheat seed before sowing, in so far as details regarding these factors were supplied for crops diseased with *G. Saubinetii*:—

Crop preceding diseased wheat.	
Potatoes ...	59 per cent.
Sugar Beet ...	9 per cent.
Lea	9 per cent.
Oats	9 per cent.
Wheat	6 per cent.
No details ...	8 per cent.

Seed treatment of wheat before sowing.	
Agrosan	18 per cent.
Ceresan	5 per cent.
Mercurial dust unspecified ...	5 per cent.
Copper Sulphate ...	9 per cent.
Tar and Lime ...	5 per cent.
No treatment ...	32 per cent.
No information ...	26 per cent.

Losses due to the Disease.

As no information on reduction in yield of grain due to this disease is available in these islands, arrangements were accordingly made to obtain actual yields from some of the diseased fields. The following three examples are from the east coast, midlands, and south-west of the country, respectively. The variety grown in all three cases was Queen Wilhelmina, preceded by a root crop, viz., sugar beet in Co. Westmeath and potatoes in both the other counties.

Table I.—Yield of Grain from Crops attacked by *Gibberella Saubinetii.*

County.	Area under wheat. Statute acres.	Yield of grain. Stones.	Calculated yield per statute acre. Stones.
Co. Louth	0 8	100	125
Co. Westmeath ...	2·2	217	98½
Co. Kerry	1 8	130	72

In the case of the Co. Westmeath crop the bushel weight of grain was only 41 lb., whereas the merchants' range of bushel weights for the same district as that in which the outbreak occurred ranged from 48 lb. (one only) to 61 lb., with an average of 56 lb. In fact, so poor did this crop appear that the first visiting mill-owner declined to thresh it, as he charged on the barrel rate.

If 160 stone (one ton) be taken as an average yield per statute acre, a figure which is not excessive, then it is obvious that serious losses occurred, the reduction in yield of grain being 21·8, 38·4, and 55 per cent., respectively, in these three cases. The actual reduction in weight of grain does not, however, represent the total loss incurred, as an examination of 500 grains taken at random from each of these crops showed. In that from Co. Louth 22 per cent. were visibly affected by the fungus, the majority of them showing more or less pink discoloration; in the sample from Co. Westmeath discoloured grains totalled only 6 per cent., but shrivelled grains amounted to 44 per cent.; and in wheat from Co. Kerry 27 per cent. showed evidence of being infected.

Description of the Disease on Wheat.

The Disease on Wheat Ears.—While the fungus *G. Saubinetii* may attack any part of the wheat plant, it is that phase of the disease on the ears which causes the greatest loss. Infection of the ears may take place from the flowering period onwards throughout the time the grain is in the stooks. The most susceptible time, however, and that during which the worst damage is done, appears to be from flowering until the grain is in the soft dough or "setting" stage. Any part of the wheat head may be attacked, and usually a single spikelet is affected first. The earliest sign of infection is the appearance of a small brown or water-soaked spot on the outer glume, which soon bleaches, and under favourable climatic conditions the disease spreads to the entire spikelet. Affected areas dry out, take on a ripened appearance, and the diseased spikelets stand out in marked contrast to the healthy green spikelets. The glumes of an infected spikelet may remain closed, being held together by the fungus growth within, and a superficial pink growth of the fungus may be evident at the base of the glumes. Under moist conditions a complete weft of whitish or pink mycelium may develop over the entire spikelet (Fig. 1, Plate 4). Depending upon dry weather conditions, a single spikelet only may be affected, and the progress of the disease stopped by lack of moisture, but in wet seasons, as in 1942, the disease spreads to spikelets above and below the original point of infection, giving rise to a group of diseased spikelets. In numerous cases examined in 1942, the central portion of the head was the part most commonly attacked, although groups of infected spikelets also occurred on the upper and lower parts of the heads. Occasionally, the whole head was diseased, but more frequently a single group of diseased spikelets only was present. Very often, however, the injury to the whole head was out of all

proportion to the actual number of diseased spikelets. This was owing to the fungus invading the rachis of the head at the base of an early infected spikelet, this invasion stopping all water and food supplies to that part of the head above the point of attack. Heads, or portions of heads, which have been killed by early seasonal attacks in this way soon become bleached, and take on the appearance of premature ripening. Nevertheless, later in the year towards harvest, owing to weathering the same heads are darker in colour than the golden healthy ones. Furthermore, such badly diseased heads remain erect when healthy heads are turning downwards under the weight of ripening grain. The contrast towards harvest time between late infected spikelets and healthy ones in the same head is not so obvious as in the case of the early infections, the only evidence of disease as a rule being the presence of a pinkish mycelium at the base of diseased glumes during damp weather.

All investigators are agreed that the grains themselves are frequently invaded. Examination of affected heads after harvest in 1942 showed all stages of the disease to exist. In some, the whole head was much smaller than normal and many of the spikelets were completely barren, the remainder enclosing a few shrunken grains. In others, though grain was present in the majority of the spikelets, it was undeveloped and shrivelled (Fig. 9, Plate 5), and showed more or less pinkish discoloration, while a felty growth of mycelium was present on the surface of some of the grains. A third type had plump firm grain in the lower part of the head, but all above a certain point on the rachis was shrunken. Finally, in a minority of cases, all grains present in the head were apparently well developed, but a few showed reddish discoloration along the groove or at the embryo end. The foregoing are evidently typical results following the time infection of the head occurred; the first representing early infection at flowering, and the last late infection when grain was almost mature, the others being intermediate stages. Infection of individual grains varies from slight to that of complete permeation by the fungus and destruction of the embryo. All shrivelled grains in an infected head are not necessarily diseased, as this depends on their proximity to the point of infection on the rachis. On the other hand, normal-looking seed taken from a diseased head may be infected by the fungus, although no discoloration is visible.

The pink or reddish discoloration was very prominent on many samples of diseased grain in 1942, the colour being most intense at the germ-end, and varying from "rose pink" to "rose colour" according to Ridgway (24). Very often a pink colour was also present inside the glumes. Although a partially coloured caryopsis was the rule, one sample was received consisting of 160 threshed grains which were coloured all over, and had the appearance of having been dipped in red ink or an eosin solution. As these grains were well developed, and, as no complaint had been made by the correspondent about shrunken grains, this sample was probably the result of late infection occurring either in the stook or stack. Bennett (5) has pointed out the very favourable conditions existing in stooks for the spread of this disease, and the prolonged

harvesting operations in 1942 certainly provided the requisite factors for infection of healthy grain.

Conidial stage.—Microscopical examination of diseased grain and glumes showed that in some cases mycelium and conidia were rather sparse, but in others both were quite abundant. The conidia were of the *Fusarium* type, the number of septa varying from three to six, with five most common where conidia were numerous. Measurements of the conidia agreed in general with those recorded by Bennett (4) for conidia of *Gibberella Saubinetii.* The conidia serve for the rapid spread of the disease throughout the growing season, and, while the conidial stage of the fungus is the one that is most frequently observed, under certain climatic conditions in autumn perithecia containing asci and ascospores also develop on infected plants. These latter fructifications occurring on debris from diseased plants provide one of the methods by which the fungus is enabled to survive the winter.

Perithecial stage.—It has already been mentioned that wheat heads which have been killed by early seasonal attacks of *G. Saubinetii* are darker than healthy heads coming on to harvest, and in wet seasons blackening of wheat ears due to various moulds is not uncommon. Perithecia on infected heads, however, in 1942 presented an appearance totally unlike that of any other disease. At a casual glance the effect was as if a very black smut was being liberated from some of the flowers, and contaminating the surrounding group of spikelets (Fig. 2, Plate 4). This was the common picture of diseased heads. Closer investigation showed that the inky-black dots and blotches consisted of fructifications, either singly or so numerous as to form crusts on the outside of the spikelets (Figs. 3 and 4, Plate 5). Microscopical examination proved the perithecia to be those of *Gibberella Saubinetii* (Mont.) Sacc. They were produced chiefly on a plectenchymatic stroma which was usually well developed. The perithecia were mainly of the ovoid verrucose type, as described by Wollenweber (33) and by Bennett (4) (Fig. 5, Plate 5), though squat forms occurred. The majority of the perithecia were mature, and contained numerous eight-spored asci, the ascospores being four-celled as a rule, although occasional uniseptate ascospores were seen. The limits of height for mature perithecia were—min. 160μ, max. 300μ; and of width, min. 160μ, max. 251μ. As can be seen from these measurements there was great variation in size of the perithecia. Where they occurred singly, as in Fig. 3, Plate 5, perithecia were on the whole larger than when they were crowded together on the stroma. On bearded varieties of wheat, like April Red, perithecia developed along the awns as well as on the glumes.

Notwithstanding the profusion of perithecia during the year 1942, none occurred on the actual wheat caryopsis itself. Some attention was paid to this point, and not only was grain from spikelets covered with perithecial fructifications scrutinised, but many pounds of diseased grain were examined with negative results. In addition, suspicious wheat grains were received several times from Dr. Lafferty, of the Seed Testing Station, Dublin, and,

although on two occasions perithecia of *G. Saubinetii* were found on fragments of glumes in the sample, in all cases the black dots which were present on grain proved to be clusters of fungal hyphae and conidiophores, a species of *Alternaria* being common. In the case of wheat, therefore, the occurrence of perithecia on the actual caryopsis itself does not appear to take place under field conditions. It is true that Bennett (4) reported the formation of perithecia on wheat grains, but the conditions under which they developed were somewhat artificial.

Gibberella Saubinetii on Oats.

Oat panicles, belonging to the varieties Marvellous and Victory II, bearing mature perithecia of *Gibberella Saubinetii* were forwarded by Mr. H. O'Donnell, Agricultural Instructor, Co. Galway. In the case of Victory II, Mr. O'Donnell reported that although he could find plenty of infected heads throughout the crop the disease on all of them was slight, and furthermore the disease was worst on that part of the field where lodging had occurred.

Owing to the open nature of the oat panicle, fungus infection seldom spreads to the central axis, therefore there is no cutting off of food supplies from healthy spikelets, and the disease is not nearly so destructive as in the case of wheat. In the specimens examined scattered infected spikelets were more common than groups of diseased spikelets, but occasionally several spikelets on a branch of the panicle showed infection as in Fig. 10, Plate 4. Nevertheless, even where infection was as bad as this on a branch of a panicle, the remainder of the head was frequently healthy.

The two samples referred to above were the only samples of infected oats received, but they constitute the first record of perithecial formation of *Gibberella Saubinetii* occurring naturally on oats in these islands. As perithecia were relatively abundant, it is probable that further investigation will prove the disease to be more widespread on oats than this single record indicates.

McInnes and Fogelman (19) reported oats to be only slightly susceptible to *G. Saubinetii*. This conclusion, however, may have been due to the particular strain of the fungus studied. Chih Tu (7) found certain strains of *G. Saubinetii* to attack oats worse than wheat; and in Finland, Rainio (23) reports oats as being badly infected.

Climatic Conditions and Development of the Disease in 1942.

In County Dublin, that period of 1942 extending from April to September, inclusive, was not only the wettest, but had the least sunshine for any similar period during the past eleven years. Rainfall in May was over 4 inches, but the precipitation in June was only 0·12 inch, which was distributed over four days, hence the month of June was dry. This, however, was the only fine summer weather during the year. From the time wheat was flowering or just

setting its grain at the beginning of July, climatic conditions were bad, the weather being dull, cloudy, and wet, and as the season advanced they became worse. Similar weather occurred over the greater part of Eire, but with considerably more rain elsewhere, see Table 2. On the east coast the daily maximum shade temperatures for July varied from 64°–74° F. with a mean of 67·7° F.; in August 61°–74° F. with a mean of 66·7° F.; and in September 56°–73° F. with a mean of 63·6° F. The daily minimum shade temperatures for the same months were: July 43°–61° F., mean 52·4° F.; August 45°–60° F., mean 53 5° F.; and September 34°–56° F., mean 49·1° F.

The humid conditions existing throughout the greater part of the growing season evidently favoured the development of *G. Saubinetii*, both the conidial and perithecial stages being abundant. Judging from the epidemic of the

TABLE 2.

Number of days on which precipitation occurred, and rainfall for each month, April to September, inclusive, 1942, at Botanic Gardens, Glasnevin, Co. Dublin, and Agricultural Stations at Ballyhaise, Co. Cavan, Athenry, Co. Galway, Clonakilty, Co. Cork.

	April		May		June		July		August		September		Totals	
	No. of days	Rainfall inches	No. of days	Rainfall inches	No. of days	Rainfall inches	No. of days	Rainfall inches	No. of days	Rainfall inches	No. of days	Rainfall inches	No. of days	Rainfall inches
Glasnevin, Co. Dublin. (East coast)	13	1·12	18	4·20	4	0·12	19	2·14	22	3·76	18	4·48	94	15·82
Ballyhaise, Co. Cavan. (North midlands)	10	2·08	15	4·62	4	0·12	19	3 51	21	4 95	23	4 83	92	20 11
Athenry, Co. Galway. (West coast)	8	2·29	16	4·66	3	0·25	23	4 54	20	4 24	17	5 10	87	21 08
Clonakilty, Co. Cork (South coast)	16	1·95	21	4·75	4	0·51	16	2·69	17	4·36	19	2 92	93	17 18

disease and the abnormally cloudy wet weather, rainfall appeared to be the chief meteorological factor concerned, not only in the degree of infection and progress of the disease afterwards, as is borne out by all investigators, but also in the development of the perithecia. Dickson, Johann, and Wineland (11) have also suggested that perithecial development is closely associated with moisture.

EVIDENCE OF *GIBBERELLA SAUBINETII* BEING ENDEMIC IN EIRE PRIOR TO THE EPIDEMIC OF 1942.

Mention has already been made of the isolation of *G. Saubinetii* by Dr. Lafferty from wheat grown in Co. Galway in 1918, and some evidence will now be presented to show that the fungus has been endemic in certain districts for some time.

The Department of Agriculture received a sample of badly diseased wheat from Co. Clare in the month of August, 1930. When this wheat was examined by the Plant Pathological Division a *Fusarium* was found to be present, but the particular species was not identified. On carrying out feeding tests with

pigs and the diseased grain at that time, the Department of Agriculture found that such grain interfered with the normal development of the animals. Some of this wheat, which was kept by the Department of Agriculture, was re-examined by the writer in November, 1942, and many of the grains showed a carmine discoloration along the groove and at the embryo end. Attempts, however, during the past winter to isolate a fungus from the discoloured grains were all negative, the grains being sterile. This is not surprising, as Shands (28) found that in the case of barley grains affected with *G. Saubinetii* the fungus was not viable after 30 months, and Christensen and Kemkamp (8) report that plating tests of three-year-old diseased grains proved the fungus to be dead. The whole appearance of the diseased grain, toxic properties, and the presence of a *Fusarium* when first received, suggest that the fungus present originally was probably the conidial stage of *Gibberella Saubinetii.* Numerous diseased specimens were received from Co. Clare in 1942, and reports from both Mr. T. Healy and Mr. T. F. Murphy, Agricultural Instructors, show that the disease was widespread in the county during the past season. Mr. Healy reported that he had observed the disease several times since 1930; and Mr. Murphy stated that infection was very noticeable on the vast majority of crops inspected in west Clare in 1942, the disease being prevalent on February sowings of winter varieties of wheat.

Reports to hand from other counties also indicate that the disease is not by any means new. In Co. Galway (where the fungus was found twenty-four years ago) Mr. H. O'Donnell, Agricultural Instructor, reported that he could find traces of the disease in almost every haggard visited during the past year. The disease has been under the observation of Mr. M. Hession, Agricultural Instructor, Co. Sligo, for the past 10–12 years, but he writes that it never was so bad as in 1942. The foregoing three counties are all on the western seaboard, but the disease has not been absent from the east coast. Mr. R. McIvor, Agricultural Instructor, Co. Louth, states, that in the case referred to in Table 1, the farmer noticed the disease three years ago, a small sheltered portion of the crop being then affected.

All observations on growing crops previous to 1942 refer of course only to a *Fusarium* stage, which may have included several species. While this may be so, it is very unlikely that *Gibberella Saubinetii* was absent and then in 1942 became the predominant fungus on wheat heads, and it is more reasonable to assume that the fungus has been endemic in certain districts for a long time.

Observations from the Seed Testing Station, Department of Agriculture, Dublin.

To begin with, it should be understood that these observations have been made quite independently of the author's own investigations. They are included here with the permission and approval of Dr. H. A. Lafferty, Head of the Seed Testing Station, to whom the writer is deeply indebted both for the

figures given herewith and for many samples of diseased grain during the past season.

After the harvest of 1938 (which incidentally followed a wet summer) attention began to be paid to a red fungus which was occurring on wheat samples undergoing germination tests in the Seed Testing Station. It is probable that the fungus occurred previously to 1938, but was not sufficiently prevalent on samples to attract undue notice. Apart from identifying the fungus as a *Fusarium* spp. no further investigation of it was undertaken except to record it on the germination sheets as the "Red Disease" on all samples where it occurred. Table 3 shows the percentages of so-called "Red Disease" recorded since the harvest of 1938 until the time of this paper going to press.

TABLE 3.

Year.		No. of Wheat Samples tested.	No. showing "Red Disease."	Percentage of Samples showing "Red Disease."
1938/'39	..	1874	12	0·6
1939/'40	...	5261	146	2·7
1940/'41	...	5658	140	2·4
1941/'42	...	7347	173	2·3
1942/'43	...	4863	1232	25·3

The first year in each case is the harvest year.

It is obvious from Table 3 that there was an exceptional development of the "Red Disease" on wheat crops last year. Since September, 1942, samples of wheat with this disease have been received from all of the 26 counties in Eire. In some cases 50 affected samples have come from a single county. The figures in Table 3 do not give any indication of the severity of the disease on the samples, but simply show its prevalence. Some of the samples of grain had only 1 or 2 per cent. of the disease present, but others were extremely bad. During the past winter severely infected samples showed as low germination as 29 and 33 per cent.

All of the infections shown in Table 3 are not necessarily caused by the fungus *Gibberella Saubinetii*, but, as it has been found repeatedly on wheat samples received recently from the Seed Testing Station, the present indications are that it is one of the principal species responsible for the so-called "Red Disease."

"SEEDLING BLIGHT."

Blight of cereal seedlings due to *Gibberella Saubinetii* has not yet been reported in this country, and to what extent this fungus may be involved in causing thin stands of wheat is not at present known.[1] That *G. Saubinetii*

[1] Since this paper was sent to the printers a number of cases of "Seedling blight" due to *Gibberella Saubinetii* have already come to hand from several counties. Practically all these cases have been on February sowings of the variety Queen Wilhelmina.

attacks wheat seedlings readily and extensively has been proved many times, and amongst the numerous investigators who have studied this problem the following consider this fungus to be a virulent parasite on wheat roots:—Adams (1), Atanasoff (2), McInnes and Fogelman (19), Dickson (10), and Henry (16). Bennett (5) also reports the fungus as capable of attacking the root system. "Seedling blight" may develop either from the fungus present in contaminated soil or from infected grains, but the latter appears to be the more common origin. Atanasoff (2) points out that an important source of infection is the seed used for sowing; and Nelson (21) states that after the wet season of 1928 many farmers in Michigan, U.S.A., were obliged to use scabbed seed, and a considerable amount of seedling blight occurred in 1929.

The symptoms of this phase of the disease depend upon the part of the seedlings attacked and the environmental conditions, Dickson (10). Thin brairds may result from sowing naturally infected seed, some of which may not be viable at all depending upon the degree of infection, or many of the germinating seedlings may be killed before they appear above ground. In less severe attacks seedlings emerge, but are weak and chlorotic, and they may succumb up to the development of the second leaf. Browning and rotting of the roots occur, the fungus mainly attacking and destroying the central cylinder first and the cortex afterwards, according to Henry (16). Congested growth of the fungus also takes place in the crown of the plant, and this, together with reduced root system, causes the death of seedlings, Bennett (5). Beyond the second leaf stage many affected plants apparently recover and produce normal heads of grain. However, depending upon the intensity of basal invasion and environmental conditions, affected plants may develop more or less "foot rot," dwarfing, and premature ripening, Bennett (5).

According to Dickson (10) the temperature of the soil is the most important single factor determining the extent of "seedling blight." For the development of the blighting of wheat he found the most favourable soil temperatures to range from 12° to 20° C. Gäumann (15) has also shown that temperature is an important factor in the invasion of wheat seedlings by *G. Saubinetii.* While a relatively high temperature is favourable for the disease, Dickson (10) found that low soil moistures accompanying low temperatures also favoured its development. That infection can take place at low temperatures is likewise shown by Bennett's investigations; he found that wheat seedlings became infected when the temperature of the greenhouse did not exceed 10° C. Furthermore, Bennett reports that "seedling blight" developed worst under conditions adverse for the host, i.e. insufficient soil moisture or water-logging.

The fungus grows up the stems of affected plants for some distance, but it is not considered systemic by the majority of investigators. Infection of the ears is derived mainly from wind-borne conidia or ascospores alighting on the anthers and glumes. The occurrence of diseased spikelets in groups at various parts of the head with healthy intervening spikelets indicates aerial infection rather than internal mycelium.

Summary of Our Knowledge on the Toxic Properties of Diseased Grain.

As the utilization of diseased grain is always an important economic question, and especially so under the present war-time conditions and scarcity of feeding stuffs, it is considered desirable to include the following summary in the present paper.

Amongst the numerous fungi which attack cereal heads *Gibberella Saubinetii* is the most serious of those producing toxic substances in affected grain. The injurious substances are transmitted to the manufactured product, and symptoms of poisoning and intoxication follow the consumption of bread made from such grain. The disease has long been familiar to Chinese farmers in Eastern Siberia, and the so-called "inebriating" bread is well known in various parts of the Russian Empire, Shapovalov (27), Dounin (13).

Following an epidemic of *Gibberella Saubinetii* in North America in 1928, and the subsequent importation of large quantities of diseased barley into Europe, severe poisoning of pigs occurred over considerable districts in Northern Germany and in parts of Holland. This led to intensive studies on this problem both in Europe and America.

Roche, Bohstedt, and Dickson (25) report that pigs, horses, and dogs, as well as man, are very sensitive to low percentages of scabbed grain, but that infected barley may be fed economically to cattle, sheep, and poultry. Mains, Vestal, and Curtis (18) found that diseased barley may be given to hogs when grain does not exceed 10 per cent. of the total ration. Barley containing up to 58 per cent. of scab was successfully fed to cattle as 50 per cent. of the grain ration, and was utilizable to the extent of 20 per cent. of the poultry ration. Mundkur and Cochran (20) reported extensive poisoning of hogs and poultry in Iowa by consumption of barley affected with *Gibberella Saubinetii* where perithecia were present on the surface of 4 to 8 per cent. of the grain. In feeding tests they found that the effects of diseased grain were variable on poultry. Young chickens fed on scabby grain developed rough plumage and lost weight, but mature birds showed no ill effects. That poultry can tolerate considerable amounts of diseased grain is also shown by the work of Titus and Godfrey (31). Rainio (23) in Finland found oats infected by *G. Saubinetii* injurious to pigs and horses when infection exceeded 20 per cent. of the grain; between 10 and 20 per cent. infection, the food was unpalatable or eaten reluctantly with consequent digestive troubles. Cattle showed no loss of appetite unless the infection exceeded 20 per cent. Christensen and Kemkamp (8) proved that scabbed wheat when fed to pigs caused the same harmful effects as similar quantities of scabbed barley. These two authors give a good clinical description of affected animals, and state that with 10 per cent. of the grains diseased the pigs did not thrive, and that barley naturally infected with 16 per cent. scab was extremely toxic to pigs.

Beller and Wedemann (3) believed the noxious principle to reside in certain organic compounds which are not susceptible to chemical analysis. Dickson *et al.* (12) considered the active substances to be apparently associated with factors containing glucosides or basic nitrogen compounds. The investigations of Schroeter and Strassberger (26) indicated that the symptoms of poisoning in animals are induced by cholin or readily hydrolysable fatty acid esters of cholin. Popp (22) attributed the poisoning properties to chemical changes in composition of the barley, the carbohydrates and albumins being partially disintegrated and toxalbumin or related toxic nitrogenous compounds formed, which caused sickness in pigs and also in poultry. Hoyman (17) found that extracted toxic substances were resistant to drying and partly inactivated by autoclaving: analysis indicated the presence of nitrogen but no sulphur or halogens.

The toxic properties of diseased grain may be reduced somewhat by prolonged storage with repeated shovellings, or by soaking and washing with water. In general the work of these investigators has shown that grain badly infected with *Gibberella Saubinetii* is not a satisfactory food for pigs and horses. It may, however, be fed to cattle, sheep, and poultry when mixed with other grains.

DISCUSSION.

The fungus *Gibberella Saubinetii* (Mont.) Sacc. has been reported from Europe, North and South America, Asia, Africa, and Australia. It attacks plants belonging to many genera and widely separated families (2), but it is most destructive on the Gramineae, cultivated cereals suffering severely. The fungus is apparently co-existent with wheat throughout its range of cultivation. In many countries the disease is relatively unimportant, but in others it is endemic in certain districts, giving rise to regular epidemics when climatic conditions are favourable.

The common name of the disease, "Ear Blight," as used in these islands, is a designation applied to several species of *Fusarium*. In America, "Wheat Scab" (1, 19, 21), "Fusarium-Blight" (2), and "Headblight" (7) are all in common use. I have been unable to ascertain when, or by whom, the term "scab" was first given to the disease on wheat. McInnes and Fogelman (19) state that Smith in 1884 named it "Wheat Scab." This statement, however, is not correct, as an examination of Smith's book (30) shows no mention of scab, although he apparently first described the blighting of wheat heads. Atanasoff (2), in his remarks on the common name of the disease, points out that in America "the head-blighting of cereal crops is generally known under the faulty name of "wheatscab"; and he goes on to say, "It is not a wheat disease alone, because it also occurs on spelt, rye, barley, oats, and certain grasses. And it is not 'scab' because it causes no scabbing of the heads or any part of the various hosts, but rather blighting of the heads." With this

last sentence the present writer is not in agreement. The perithecial stage of the fungus as it occurred in Eire in 1942 consisted of definite incrustations on the spikelets, giving them a scabbed appearance, Fig. 2, Plate 4, and Figs. 3 and 4, Plate 5, and "Wheat Scab" or—as the disease occurs on other cereals—preferably "Cereal Scab" is a very appropriate name for this phase of the disease.

Perithecial formation on the heads of both wheat and oats is so distinctive that it is rather remarkable there is no previous record of its occurrence in this country. It might be argued that owing to the present demands for extensive cereal cultivation, a much larger acreage is now under these crops than formerly, and hence, any disease would be more readily noticed. While this is partly true, wheat was grown in the past to some extent every year, and considerable areas of oats and barley have always been cultivated in this country. Granting that perithecia do not develop every year, wet seasons have not been infrequent, yet there is no account of anything resembling perithecial formation on any cereal crop. In the case of barley in particular, any outbreak of *G. Saubinetii* on the grain, either with or without perithecia, could scarcely have been overlooked, as the presence of the fungus on this cereal is stated to interfere with the malting quality, Shands (29). The epidemic on wheat in 1942 may have been due to a particular strain of the fungus which attacks wheat more readily than other cereals, but this point requires further investigation. Chih Tu (7) has shown that different strains of *G. Saubinetii* exist, varying in their virulence on wheat, barley, and oats, and also in their capacity to attack different varieties of the same host. None of the hosts mentioned, however, were immune to attack by any of the strains isolated. The pathogenicity of strains was also investigated by Ullstrup (32) and by Eide (14), the latter reporting difference in ability of strains to produce perithecia.

Owing to the fact that perithecia develop abundantly on stubble of affected crops, as well as on the ears, in countries where the disease is prevalent rotation of cereal crops is strongly recommended as one of the control measures. As wheat is just now being grown occasionally two or more years in succession, and under the present exigency frequently follows other cereals, it was thought that this might have been a predisposing factor in the epidemic of 1942. There is no evidence, however, that such was the case. From the data given, it is apparent that the disease was prevalent on wheat following root crops, occurring after roots in 68 per cent. of the cases reported, and in 59 per cent. of the cases potatoes constituted the previous crop. *Gibberella Saubinetii* is not known to occur as a regular parasite of potatoes, although Wollenweber (33) records isolating this fungus from potato berries. The severity of the disease on wheat sown after root crops was probably associated with the more luxuriant foliage of the wheat after roots rather than to any infection arising from the previous crop. Atanasoff (2) found that on healthy, vigorous, and more succulent plants the infection spreads more rapidly than on plants of average vigour.

Wheat from badly affected crops should not be used for seed purposes. It is evident that the germination of such seed will be low to begin with; secondly, its use may result in "seedling blight," or diseased plants may serve as a source of infection to the crop in general; and thirdly, it is clear from data given that seed dressings do not control this disease effectively.

The author is indebted to the Department of Agriculture, Dublin, for every facility in carrying out these investigations, and also to the various County Instructors in Agriculture who not only forwarded specimens for examination but unreservedly supplied many observations on the disease. Grateful acknowledgement is also made to the Imperial Mycological Institute, Kew, for the loan of several Bulletins which were unobtainable in Eire.

Summary.

An outbreak of *Gibberella Saubinetii* (Mont.) Sacc. on an epidemic scale on wheat in Eire in 1942 is described.

The disease appeared during the flowering period of the host, and in some cases was so severe that many wheat heads had no fully developed grains, the flowers either being empty or having shrivelled seed. From 0·8, 2·2 and 1·8 statute acres the actual yields of grain were 100, 217 and 130 stones, respectively. Reduction in yield due to the disease in these cases was of the following order:—21, 38, and 55 per cent. These figures, however, do not represent the total loss, as much of the remainder of the crop in each case consisted of shrivelled grain of low germination.

The epidemic was associated with showery weather which began early in July, and as the season advanced climatic conditions became worse. The summer of 1942 had the least sun and was the wettest in Eire for eleven years.

Perithecial development occurred freely on several varieties of wheat and was recorded from ten separate counties. It is believed that moisture rather than temperature was the main factor responsible for perithecial formation.

Perithecia of *G. Saubinetii* were also found on two varieties of oats. This apparently constitutes a first record of these fructifications occurring naturally on oats in these islands.

Some evidence is presented to show that the disease has been endemic in certain districts for a considerable time.

Owing to the scabbed appearance of spikelets of wheat and oats with perithecia of *G. Saubinetii* on them, "Cereal Scab" is quite an appropriate name for this phase of the disease.

EXPLANATION OF PLATES.

Plate 4.

Fig. 1.—Healthy wheat head on left, two diseased heads on right showing general effects of attack and weft of mycelium of *G. Saubinetii* on affected spikelets. Variety Queen Wilhelmina.

Fig. 2.—Perithecia of *G. Saubinetii* on wheat ears, affected spikelets occur in groups. Variety Q. Wilhelmina.

Fig. 10.—Branch of oat panicle showing perithecia of *G. Saubinetii* on two spikelets. × 3.

Plate 5.

Figs. 3 and 4.—Spikelets removed from diseased wheat heads, showing medium and prolific development of perithecia of *G. Saubinetii.* × 3.

Figs. 5 to 8.—Photomicrographs. Fig. 5.—Median longitudinal section through perithecium of *G. Saubinetii* showing general appearance. × 200. Fig. 6.—Section through perithecium but not median, showing proliferation of cells around the neck of the fructification. Section squashed to show numerous asci and paraphyses. × 200.

Fig. 7.—Mature ascospores in ascus. × 750.

Fig. 8.—General appearance of ascospores when removed from ascus. × 1000.

Fig. 9.—Wheat grains from affected ears on left, healthy grains on right.

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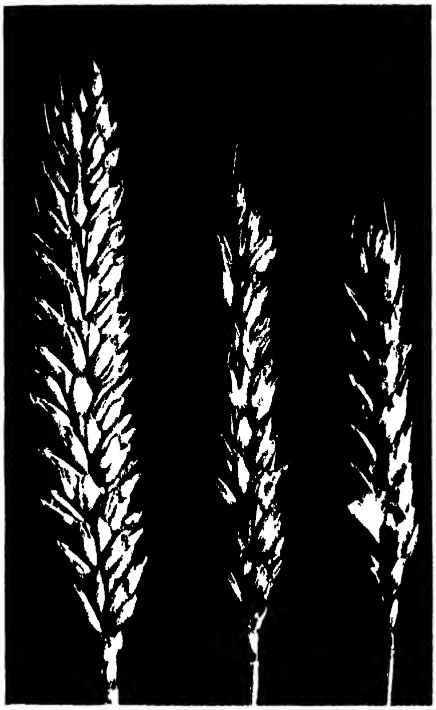

FIG. 1.

FIG. 2.

FIG. 10.

Fig. 3.

Fig. 4.

Fig. 5.

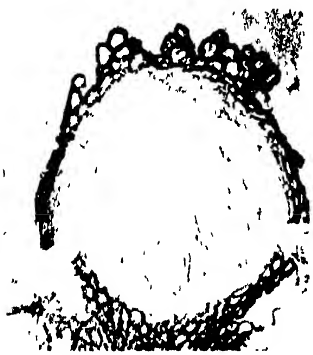

Fig. 6.

Fig. 7.

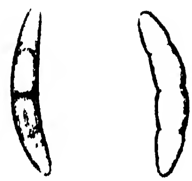

Fig. 8.

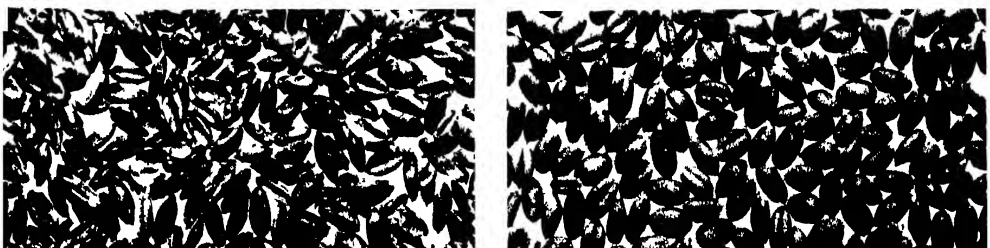

Fig. 9.

Photos] [*G. H. McLean*

THE

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Vol. 23 (N.S.).

[SEPARATE ISSUE.]

Nos. 12 and 13. July, 1943.

STUDIES ON UREIDES. PART I. PREPARATION AND HYDROLYSIS OF DIACYL UREAS FROM ALIPHATIC ACIDS.

By A. E. A. WERNER.

THE ACTION OF THIONYL CHLORIDE ON UREA AND URETHANE.

By E. A. WERNER and A. E. A. WERNER.

Price One Shilling.

No. 12.

STUDIES ON UREIDES. PART I PREPARATION AND HYDROLYSIS OF DIACYL UREAS FROM ALIPHATIC ACIDS

By A. E. A. WERNER.

[Read May 25 Published separately July 1, 1943.]

The symmetrical diacyl derivatives of urea are usually represented by the normal carbamidic NN′ structure in which both acyl groups are attached to nitrogen atoms; but E. A Werner (1) has suggested that the second acyl group introduced into the molecule is attached to oxygen to form a pseudo-ureido ester, basing this structure on the behaviour of diacetyl urea when heated and when subjected to aqueous hydrolysis These two possible structures may be represented by the following formulae A and B.—

$$(A) \quad O = C\begin{matrix} \diagup NH - CO - R \\ \diagdown NH - CO - R \end{matrix} \qquad (B) \quad HN = C\begin{matrix} \diagup NH - CO - R \\ \diagdown O \cdot CO - R \end{matrix}$$

The present communication is concerned with the preparation of diacyl ureas, containing two different acid radicals, since the properties of such compounds, in particular their behaviour on hydrolysis, may be expected to provide the necessary experimental evidence to distinguish conclusively between the two alternatives. If structure B correctly represents the diacyl ureas, then it ought to be possible to obtain two isomeric derivatives, namely.

$$(B_1) \quad HN = C\begin{matrix} \diagup NHCOR \\ \diagdown O - COR_1 \end{matrix} \qquad \text{and} \qquad (B_2) \quad HN = C\begin{matrix} \diagup NH \;\; CO - R_1 \\ \diagdown O - CO - R \end{matrix}$$

depending upon the order in which the acyl groups are introduced into the molecule, whereas structure (A) can only yield one compound independent of the order in which the acyl groups are introduced. Three such pairs of diacyl ureas, namely, acetyl-propionyl, propionyl-butyryl, and acetyl-butyryl ureas, were prepared as described in the experimental part, but in no case was there any evidence of the existence of isomeric derivatives, thus, for example, the same substance is obtained by reacting monoacetyl urea with propionic anhydride or by reacting propionyl urea with acetic anhydride.

It would therefore seem as if diacyl ureas must have the symmetrical carbimidic NN′ structure (A), a fact which would be in agreement with the preparation of diacetyl urea by the interaction of phosgene with acetamide as described by Schmidt (2).

E. A. Werner (*loc. cit.*) has shown that, whereas monoacetyl urea undergoes no appreciable amount of hydrolysis when its aqueous solution is boiled

under reflux, the diacetyl derivative readily splits off one acetyl group. On the basis of the pseudo-ureido ester structure for the diacetyl derivative this hydrolysis was represented by the following equation:—

$$HN = C\begin{matrix} \diagup NH - CO - CH_3 \\ \diagdown O - CO - CH_3 \end{matrix} + H_2O == HN = C\begin{matrix} \diagup NH - CO - CH_3 \\ \diagdown OH \end{matrix} + CH_3COOH, \quad (1)$$

since the resulting monoacetyl urea was stable towards mere aqueous hydrolysis. Upon investigating the behaviour towards aqueous hydrolysis of the other diacyl derivatives described in the present communication, it was found in all cases that, whereas the diacyl derivatives readily split off one acyl group, the monoacyl derivatives were resistant to aqueous hydrolysis. The extent of hydrolysis was followed by refluxing decimolecular solutions and titrating the liberated acid after specified intervals of time; the results so obtained are collected together in Table I.

TABLE I.—*Aqueous Hydrolysis of Diacyl Ureas.*

Nature of Acyl groups.	Percentage Hydrolysis after: 1 hour.	2 hours.	3 hours.	6 hours.	9 hours.
Diacetyl	21	32	45	71	86
Acetyl Propionyl ...	18	30	42	68	82
Dipropionyl ...	20	32	44	70	85
Propionyl Butyryl ...	15	24	35	65	80
Acetyl Butyryl ...	22	28	39	69	85
Dibutyryl ...	10	21	30	61	75

The course of the hydrolysis cannot, however, be represented by the above equation, because the product obtained by hydrolysis of those diacyl derivatives containing two different acyl groups was not homogeneous; the two acyl groups were eliminated simultaneously, so that the product of the reaction was an approximately equimolecular mixture of the two possible monoacyl derivatives. Assuming the NN′ carbamidic structure for the diacyl derivatives the course of the hydrolysis is best represented by the following equation:—

$$2\,O = C\begin{matrix} \diagup NH - CO - R \\ \diagdown NH - CO - R_1 \end{matrix} + 2\,H_2O == O = C\begin{matrix} \diagup NH - CO - R \\ \diagdown NH_2 \end{matrix} + O = C\begin{matrix} \diagup NH - CO - R_1 \\ \diagdown NH_2 \end{matrix} + R - COOH + R_1COOH \quad (2)$$

The stability of the monoacyl ureas towards aqueous hydrolysis was also confirmed by the fact that no urea is produced during the hydrolysis.

The hydrolysis of the diacyl and monoacyl ureas under alkaline conditions was next studied. It was found that both the diacyl and monoacyl derivatives were rapidly hydrolysed in the cold, the rate of hydrolysis being much greater

than in the case of aqueous hydrolysis. The actual results obtained in this series of experiments are collected in Table II.

TABLE II.—*Alkaline Hydrolysis of Di- and Mono-Acyl Ureas.*

Nature of Acyl groups.	Percentage Hydrolysis after: 5 mins.	10 mins.	20 mins.	30 mins.	60 mins	120 mins.
Diacetyl ...	23	33	48	55	67	75
Acetyl Propionyl ...	25	37	49	57	68	78
Dipropionyl ...	27	40	54	60	65	78
Propionyl Butyryl ...	25	36	49	58	64	74
Acetyl Butyryl ...	24	34	46	56	65	76
Dibutyryl ...	22	32	48	55	65	77
Acetyl ...	17	34	42	57	—	71
Propionyl ...	19	38	49	56	63	—
Butyryl ...	21	37	49	58	65	—

The monoacyl ureas were hydrolysed using one molecular proportion of sodium hydroxide, but in the case of the diacyl ureas two molecular proportions of alkali were used so as to cause complete hydrolysis. In the latter case the presence of free urea in the solution was tested for at frequent intervals, but the presence of urea was not detected until at least 50 per cent. hydrolysis had occurred, i.e. until the diacyl derivative had been completely converted into the monoacyl derivative. This observation confirms the fact that the diacyl ureas are more susceptible to hydrolysis than the monoacyl derivatives, and secondly that the hydrolysis proceeds in an asymmetrical manner as indicated in equation 2. The two acyl groups are not simultaneously removed with the immediate formation of urea from the diacyl derivative as would be represented by the following equation 3:—

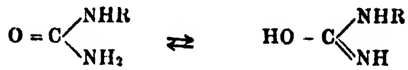
$$O = C\begin{matrix}\diagup NH - CO - R \\ \diagdown NH - CO - R_1\end{matrix} + 2H_2O = O = C\begin{matrix}\diagup NH_2 \\ \diagdown NH_2\end{matrix} + R - COOH + R_1 - COOH \qquad (3)$$

Since equation 1, involving the pseudo-ureido ester structure for the diacyl derivatives, does not correctly represent the course of the hydrolysis in aqueous solution, some alternative explanation must be sought to account for this remarkable difference between the behaviour of mono- and di-acyl derivatives towards hydrolysis, a difference which is not to be expected by a comparison of the normal structures, namely,

$$O = C\begin{matrix}\diagup NH - CO - R \\ \diagdown NH_2\end{matrix} \quad \text{and} \quad O = C\begin{matrix}\diagup NH - CO - R \\ \diagdown NH - CO - R\end{matrix}$$

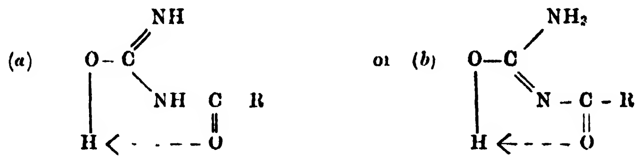
The following suggestions offer a possible explanation of the experimental observations. E. A. Werner (3) has shown that monosubstituted alkyl

derivatives of urea exist as an equilibrium mixture

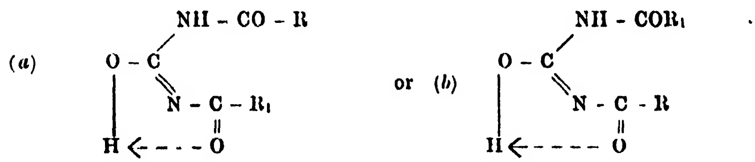


in which the relative amounts of the keto and enol tautomeric forms can be determined by the action of nitrous acid, e.g. in the case of methyl urea there is present 90 per cent. of the enol form. It is therefore probable that the acyl derivatives also exist as similar equilibrium mixtures in which the enol form will predominate to a large extent. Furthermore, the enol form will be stabilised owing to the formation of a six-atom cyclic structure due to chelation between the enolic hydrogen atom and the oxygen atom of the carbonyl group. The following equilibrium systems thus arise:—

(i) for the monoureides



(ii) for the diureides (with different acyl groups attached to the nitrogen atoms)



The formation of these stable chelate rings immobilises the active carbonyl group, thus preventing the removal of an acyl group by aqueous hydrolysis; in alkaline solution the chelate rings are destroyed prior to hydrolysis. It therefore follows that the monoacyl derivatives of urea will resist aqueous hydrolysis, but will be hydrolysed in alkaline solution; whereas in the case of the diacyl derivatives chelation can only immobilise one of the two acyl groups, so that the other acyl group is removed by aqueous hydrolysis. If there is no great difference in the character of the two acyl groups present in the molecule both will undergo enolisation and chelation to about the same extent, with the result that aqueous hydrolysis splits off both acyl groups in approximately equal amounts. This is the case with the diacyl derivatives in the present communication. The preparation and hydrolysis of mixed aliphatic aromatic diureides will be considered in a future communication.

Experimental.

Monoacyl derivatives of urea.—One molecular proportion of urea was added to one molecular proportion of the appropriate acid anhydride, containing concentrated sulphuric acid (0·5 c.c.), and a sufficient volume of the appropriate

fatty acid was subsequently added to effect complete solution. Brisk reaction started when the solution was warmed on the water-bath to a temperature ranging from 60° to 80° C., which was controlled by periodic immersion of the reaction flask in crushed ice. In order to avoid undesired side reactions accompanying the decomposition of the acetylated ureas, it is imperative that the temperature should not exceed 100° C. On completion of the reaction the solution was poured into cold water; the desired monoacyl derivatives were precipitated as white solids, which were filtered off and washed with 10 per cent. sodium bicarbonate solution.

Propionyl Urea was recrystallised from hot alcohol. White feathery crystals. M.P., 202° C. Yield, 70 per cent. of the theoretical. (Found N = 24 00 per cent. $C_4H_8ON_2$ requires N = 24·14 per cent)

Butyryl Urea was recrystallised from hot water. Fine acicular crystals. M.P., 170° C. Yield, 65 per cent. of the theoretical. (Found N = 21 60 per cent. $C_5H_{10}O_2N_2$ requires N = 21 54 per cent)

Symmetrical diacyl derivatives of urea.—These were obtained by treating the monoacyl derivatives with the appropriate acid anhydride or acid chloride, the temperature being kept as low as possible. Higher yields were obtained when the monoureide with lower molecular weight was first prepared and treated with the acid anhydride of higher molecular weight.

Acetyl Propionyl Urea was recrystallised from 50 per cent. propionic acid. White microcrystalline powder. M.P., 113° C. Yields, 80 per cent and 60 per cent. of the theoretical. (Found N = 17·55 per cent. $C_6H_{10}O_3N_2$ requires N = 17·70 per cent.)

Dipropionyl Urea was recrystallised from hot water. White pearly leaflets. M.P., 207° C. Yield, 75 per cent. of the theoretical. (Found N = 16 60 per cent. $C_7H_{12}O_3N_2$ requires N = 16 40 per cent)

Acetyl Butyryl Urea was recrystallised from petroleum ether. Glistening plates. M.P., 82° C. Yields, 80 per cent. and 65 per cent of the theoretical. (Found N = 16 50. $C_7H_{12}O_3N_2$ requires N = 16 40 per cent)

Propionyl Butyryl Urea was recrystallised from a mxiture of ethyl alcohol and petroleum ether. White glistening plates. M.P., 97° C. Yields, 75 per cent. and 65 per cent. of the theoretical. (Found N = 14·90 per cent. $C_8H_{14}O_3N_2$ requires N = 15 05 per cent.)

- *Dibutyryl Urea* was recrystallised from mixture of benzene and petroleum ether. Waxy leaflets. M.P., 92° C. Yield, 60 per cent. of the theoretical. (Found N = 13·95 per cent. $C_9H_{16}O_3N_2$ requires N = 14 00 per cent.)

Aqueous Hydrolysis of Diacyl Ureas.—Decimolecular solutions of the respective compounds were boiled under reflux. The amount of fatty acid set free during the hydrolysis is determined periodically by titrating the solution with N/10 sodium hydroxide. On cooling the solution after hydrolysis white crystalline deposits were obtained. These were identified by determining their M.P.'s and nitrogen contents. Tests for the presence of urea in the solution were carried out by the urease method, but all proved negative.

Dipropionyl Urea yielded a white crystalline product. M.P., 203° C., and N = 24 20.

Acetyl Propionyl Urea yielded a white crystalline product. M.P., 192°–196° C. N = 25 60 per cent., 26·00 per cent. Equimolecular mixture of acetyl and propionyl ureas melts at 192°–194° C. and requires N = 25·75 per cent.

Propionyl Butyryl Urea yielded a white crystalline product. M.P., 159°–165° C. N = 22·60 per cent. Equimolecular mixture of propionyl and butyryl ureas melts at 159°–164° C. and requires N = 22 84 per cent.

Dibutyryl Urea yielded a white crystalline product. M.P., 171° C., and N = 21 45 per cent.

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No. 13.

THE ACTION OF THIONYL CHLORIDE ON UREA AND URETHANE.

By E. A. WERNER and A. E. A. WERNER.

[Read May 25. Published separately July 1, 1943.]

SCHROETER and Lewinski (1) have shown that thionyl chloride and urethane interact in accordance with the following equation, leading to the formation of ethyl allophanate as the main product of the reaction:—

$$2\,O = C\begin{matrix}\diagup NH_2 \\ \diagdown OC_2H_5\end{matrix} + SOCl_2 = O = C\begin{matrix}\diagup NH_2 \\ \diagdown NHCO - OC_2H_5\end{matrix} + SO_2 + HCl + C_2H_5Cl.$$

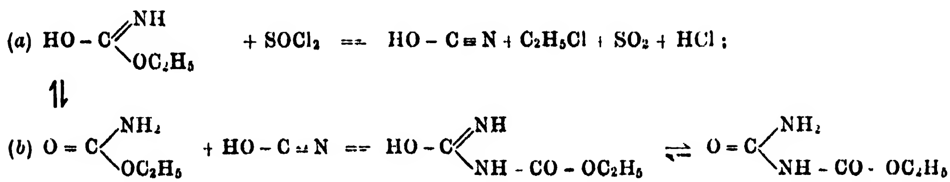
The reaction was carried out in benzene solution heated under reflux at 80° C. using two molecular proportions of urethane and one molecular proportion of thionyl chloride, and the yield of ethyl allophanate was 70 per cent. of the theoretical. This reaction was later investigated by Warren and Wilson (2), using a large excess of thionyl chloride in the absence of a solvent; they confirmed the formation of ethyl allophanate, but gave no reference to the yield obtained. The present authors find that the reaction proceeds rapidly without the use of either a solvent or an undue excess of thionyl chloride, and, as shown in the experimental part, the yield obtained under these simplified conditions is 90 per cent. of the theoretical, so that this reaction constitutes the best method for the preparation of ethyl allophanate. It has also been shown that sulphuryl chloride (3), phosgene (4), and phosphorus pentoxide (5) bring about a similar reaction, with the production of ethyl allophanate in varying amounts. The chief purpose of the present communication is to present a suitable mechanism to represent the course of these reactions supported by adequate experimental evidence.

Previous authors have simply represented the reaction as merely involving the removal of one molecule of ethyl alcohol from two molecules of urethane, without offering any explanation of the actual mechanism involved. Davis (6), in a communication on the hypothetical substance which he calls dicyanic acid,[1] represents the formation of ethyl allophanate from urethane as being due to the addition of this dicyanic acid to ethyl alcohol, according to the following equation:—

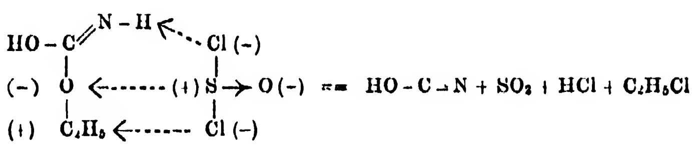
$$C_2H_5OH + H_2N - CO - N = C = O \quad = \quad O = C\begin{matrix}\diagup NH_2 \\ \diagdown NH - CO - OC_2H_5\end{matrix}$$

[1] The existence of dicyanic acid must be regarded as very doubtful; the compound has never been isolated by Davis, and the reason adduced for its existence is based on a misjudged view of an analogy between cyanic acid and cyanamide. The latter forms the well-defined dimeride, dicyanodiamide, by mutual neutralisation of the *acidic* form $HN = C = NH$ and the *basic* tautomeric form $N \equiv C - NH_2$. Both tautomeric forms of cyanic acid are acidic, and therefore the formation of a dimeride is not to be expected.

Since the experimental conditions of the reaction under discussion would not be favourable to even the temporary formation of a substance so unstable as dicyanic acid, it is highly unlikely that this substance is concerned in the formation of ethyl allophanate. Accordingly it is suggested, in agreement with the view of one of us (E.A.W.) (7), regarding the structure of urethane in an acid medium, that in all these reactions the formation of the ethyl allophanate takes place in two stages. In the first stage cyanic acid is liberated in an "active" form (the exact nature of this "active" form of cyanic acid will be discussed in a later communication) from one molecule of urethane; in the second stage addition of "active" cyanic acid to a molecule of unchanged urethane occurs. This mechanism may be represented by the following equations:—



which proceed simultaneously once the initial decomposition of one urethane molecule has started. The actual manner in which the urethane molecule is attacked by the thionyl chloride may be satisfactorily represented by the following mechanism suggested by the work of Gerard (8) on the action of thionyl chloride on hydroxylic compounds:—



(the signs in parentheses represent the tendencies indicated).

The high yield of ethyl allophanate is due to the fact that reaction (*b*) proceeds more rapidly than reaction (*a*), so that there is always unchanged urethane present in the system, with which the cyanic acid can immediately combine before it polymerises with the formation of cyanuric acid.

The analogous reaction of thionyl chloride on urea has been studied by Warren and Wilson (*loc. cit.*), who claim as the chief product of the reaction the formation of biuret, which they represent as taking place by the elimination of one molecule of ammonia from two molecules of urea; they give no figures as to the actual yield obtained. A repetition of this experiment as shown in the experimental part has proved that in actual fact the yield of biuret is very small, being of the order of 10 per cent. of the theoretical; the main products of the reaction are ammonium chloride, ammonium cyanate, and other decomposition products of cyanic acid. If this reaction merely involved the removal of one molecule of ammonia from two molecules of urea one would

expect the yield of biuret to be considerably higher. It is evident that the reaction proceeds by a similar mechanism to that suggested in the case of urethane with, however, one important point of difference, namely, the fact that the urea molecule is broken down by the thionyl chloride at a much greater rate than that at which it combines with unchanged urea to form biuret; for this reason most of the cyanic acid enters into side reactions with a corresponding decrease in the yield of biuret. This difference between urethane and urea is what would be expected, since urea breaks up into ammonia and cyanic acid at the relatively low temperature of 132° C., whereas urethane only decomposes into ethyl alcohol and cyanic acid at 184° C.

This explanation of the marked difference in these two analogous reactions receives further support from a study of the action of thionyl chloride on an equimolecular mixture of urea and urethane. There are two alternative courses that the reaction may follow, represented schematically as follows:—

A.

URETHANE at 184°C. ⟶ ETHYL ALCOHOL + CYANIC ACID ⟶ + UREA ⟶ BIURET

B.

UREA at 132° C. ⟶ CYANIC ACID + AMMONIA ⟶ + URETHANE ⟶ ETHYL ALLOPHANATE

The relative yields of biuret and ethyl allophanate will indicate the extent to which the two reactions occur. It was found that the yield of ethyl allophanate was 85 per cent. of that required according to reaction scheme B, whereas the yield of biuret was only 10 per cent. of that required according to reaction scheme A. This result indicates clearly that the urea is preferentially decomposed to yield cyanic acid, which combines with the undecomposed urethane. This idea is also supported by the experimental observation that in this reaction no ethyl chloride is produced, whereas in the reaction with urethane alone ethyl chloride distils over in copious amount as soon as the reaction starts. This complete absence of ethyl chloride indicates furthermore that the small amount of biuret formed is not produced according to reaction mechanism A, but is formed as the result of cyanic acid produced in reaction mechanism B combining with some unchanged urea, as indicated above by dotted arrow.

A final point of practical importance is the fact that cyanuric acid can be produced in quantitative yield by heating ethyl allophanate just above its melting point; it decomposes smoothly in accordance with the following equation:—

$$3\ O=C\begin{cases} NH_2 \\ NHCO-OC_2H_5 \end{cases} = 2\ C_3H_3N_3O_3 + 3\ C_2H_5OH$$

Schroeter and Lewinski (*loc. cit.*) heated ethyl allophanate with thionyl chloride in a sealed tube at 140°–150° C. using xylene as a diluent, but only succeeded in obtaining a 60 per cent. yield of cyanuric acid. If, however, thionyl chloride is allowed to react with urethane at the elevated temperature of 200° C. cyanuric acid is produced directly in one stage in quantitative yield, and this reaction constitutes the most convenient method for the preparation of pure cyanuric acid, since its formation is not accompanied by any solid by-products.

Experimental.

Action of thionyl chloride on urethane.—Urethane (27 g.) was warmed with thionyl chloride (25 g.) to 60° C., when a vigorous reaction set in accompanied by the evolution of hydrochloric acid, sulphur dioxide, and *ethyl chloride,* which were absorbed in alcoholic potassium hydroxide. After 1½ hours heating the reaction is completed, and the residue is extracted with ether (40 c.c.) and filtered. Weight, 17·7 g. Recrystallised from hot water M.P. is 190° C. (Found N = 21 09 per cent. $C_4H_8N_2O_3$ requires N = 21·2 per cent.)

Action of thionyl chloride on urea.—Urea (20 g.) was warmed to 70° C. with thionyl chloride (25 g.). Reaction occurs with evolution of hydrochloric acid and sulphur dioxide absorbed in alcoholic potassium hydroxide. Residue is extracted with ether (50 c.c.). Yield of biuret obtained was 2 g.

Action of thionyl chloride on an equimolecular mixture of urethane and urea.—A finely powdered mixture of urethane (14 g.) and urea (9 g.) was warmed to 80° C. with thionyl chloride (25 g.). Reaction occurred with evolution of hydrochloric acid and sulphur dioxide. The solid residue was extracted with ether to remove unchanged urethane, and then with hot water to remove biuret. The residue was recrystallised from water. Weight, 8 5 g. M.P., 190° C. (Found N = 21·15 per cent.) The aqueous filtrate yielded 2·5 g. of biuret and 4 g. of ammonium chloride.

Thermal decomposition of ethyl allophanate.—Ethyl allophanate (13·2 g.) was heated to 200° C. for 45 minutes. Ethyl alcohol distilled over, and a residue of pure anhydrous cyanuric acid remained. Weight, 7·85 g., corresponding to 93 5 per cent. of the theoretical.

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No. 14.

REPORT OF THE IRISH RADIUM COMMITTEE FOR THE YEAR 1942.

There has been a considerable reduction in the demand for radon, only 1,109 tubes containing 6,648 millicuries having been issued, as compared with 1,400 containing 10,613 millicuries in 1941.

Mr. M. G. Kelliher was elected to the Radium Exhibition falling vacant during the year.

Returns from the chief users record the treatment of the following cases with radon or radium element:—

Malignant Cases.

St. Anne's Hospital, Dublin	140
Dr. Oswald Murphy, St. Vincent's Hospital ..	80
Dr. Oliver Chance	14
Dr. d'Abreu, City & County Hospital, Waterford ...	10
	244

Non-Malignant.

Dr. Murphy	67	
Dr. Chance	38	
Meath Hospital	2	
Dr. Solomons	1	
	108	Total: 352

The returns show that for the cases of malignant disease first seen during 1942 the numbers treated by the different methods were as follows:—

1.	Number of cases of all types of malignant disease seen during 1942 ...	928
2.	„ „ treated during 1942	730
3.	„ „ „ by Surgery alone	54
4.	„ „ „ by Radium or Radon alone ...	184
5.	„ „ „ by X-ray alone	406
6.	„ „ „ by Surgery and Radium ...	17
7.	„ „ „ by Surgery and X-rays ...	26
8.	„ „ „ by Radium and X-rays ...	41
9.	„ „ „ by Surgery, Radium, and X-rays	2

Dr. Oswald J. Murphy [St. Vincent's Hospital, Dublin] reported the treatment with radon of the following cases:—

Malignant.—Carcinoma of the ear, 2; lip, 5; cheek, 1; parotid, 1; tongue, 3; tonsil, 1; œsophagus, 1; uterine cervix, 8; uterine body, 2; skin, 31. Rodent Ulcers, 27. Total, 82.

Non-Malignant.—T.B. Peritonitis, 10. Uterine Hæmorrhage, 4. Angiomata, 18. Lupus Vulgaris, 2. Lupus Erythematosus, 2. Keloids, 2. Warts, 29. Total, 67.

Dr. Oliver Chance [Richmond, Sir Patrick Dun's, Dr. Steevens', Rotunda, and Coombe Hospitals] reported the treatment with X-rays, radium, or radon, of the following cases:—

Malignant.—Carcinoma of the skin, 13; lip, 12; tongue, 4; palate, 1; floor of mouth, 1; fauces, 2; pharynx, 3; extrinsic larynx, 4; post-cricoid, 1; breast, 17; cervix, 6; body of uterus, 1; ovary, 1; vulva, 2; prostate, 1; lung, 1; glands (unclassified), 3. Sarcoma, 1. Brain Tumour, 1. Rodent Ulcers, 37. Total, 112.

Non-Malignant.—Toxic Goitre, 12. T. B. Peritonitis, 6. Non-malignant uterine conditions, 4. Naevi, 8. Various other non-malignant conditions, 8. Total, 38.

Dr. Bethel Solomons reported the curing by radon treatment of a case of Metropathia Hæmorrhagica.

The Meath Hospital reported the treatment by radon of one case of Toxic Goitre, and one of Hyperthyroidism.

THE

SCIENTIFIC PROCEEDINGS

OF THE

ROYAL DUBLIN SOCIETY.

Vol. 23 (N.S.).

[*SEPARATE ISSUE.*]

No. 15. August, 1943.

THE CHEMICAL CONSTITUENTS OF LICHENS FOUND IN IRELAND —*LECANORA GANGALEOIDES.*—Part 3.—THE CONSTITUTION OF GANGALEOIDIN.

By V. E. DAVIDSON, Ph.D., J. KEANE, Ph.D.,

AND

T. J. NOLAN, B.A., D.Sc.,

University College, Dublin.

Price Two Shillings.

No. 15.

THE CHEMICAL CONSTITUENTS OF LICHENS FOUND IN IRELAND —*LECANORA GANGALEOIDES*.—PART 3.—THE CONSTITUTION OF GANGALEOIDIN.

BY V. E. DAVIDSON, PH.D., J. KEANE, PH.D.,

AND

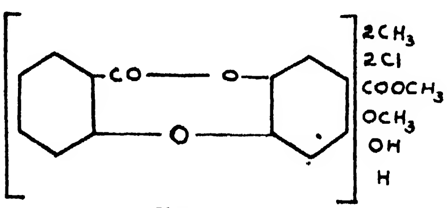
T. J. NOLAN, B.A., D.Sc.,
University College, Dublin.

INTRODUCTION.

[Read MAY 25. Published separately AUGUST 13, 1943.]

NOLAN and Keane (Sci. Proc. R.D.S., 1935, **21**, p. 141; 1940, **22**, p. 199) have described the isolation from the lichen *Lecanora gangaleoides* of atranorin and chloratranorin, arabitol, rhodophyscin, endococcin (the latter transformed into the former by boiling with acetic acid), a chlorine-containing substance which they named gangaleoidin and to which they gave the formula $C_{18}H_{14}O_7Cl_2$, and another chlorine-containing substance of much higher melting point than gangaleoidin, differing from the latter in methoxyl content, but otherwise giving analyses identical with those of gangaleoidin.

Gangaleoidin was found to contain two methoxyl groups, a hydroxyl group, and a lactone group (CO–O). One of the methoxyl groups was considered to be present as a carboxylic ester, so that the formula was written $C_{14}H_7OCl_2(COO)(COOCH_3)(OCH_3)(OH)$. General considerations of this formula and lack of reactivity of the remaining oxygen atom indicated the presence of two aromatic rings joined by an oxygen bridge. Since the chlorine atoms were not affected by boiling alcoholic potash they could not be present in an aliphatic side chain, but must be substituents in a benzene ring. General considerations of lichen chemistry led Keane and Nolan to the view that the aliphatic side chains consisted of two methyl groups and not of a single ethyl substituent. Accordingly they wrote the formula for gangaleoidin as I; they attempted by means of pyrolytic methods to effect scission of the molecule but without success. When the dicarboxylic acid II



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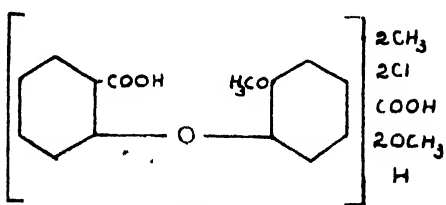
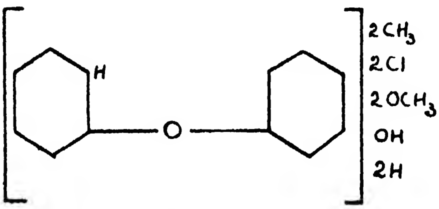


II

derived from gangaleoidin was distilled under reduced pressure three compounds were obtained. One was the monocarboxylic acid produced by partial decarboxylation; the second was a diphenyl ether of the constitution III, complete decarboxylation and partial demethylation having taken place; the third was a neutral product of formula $C_{18}H_{16}O_5Cl_2$, which contained three methoxyl groups. This product, though it did not form a salt with concentrated hydrochloric acid, is most probably a xanthone IV.



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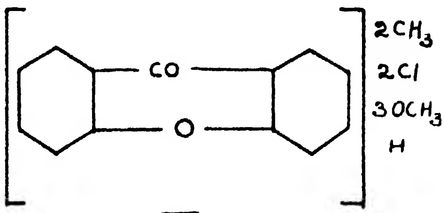
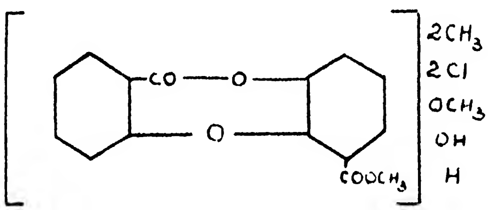


IV

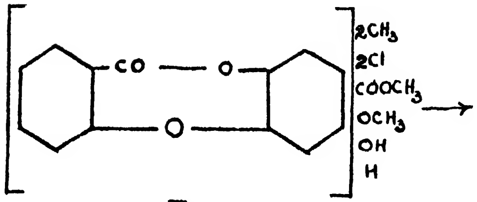
Should it have been formed without any preliminary isomerisation then the carboxylic ester group in gangaleoidin must be in the ortho position to the oxygen bridge as in V, or alternatively this ortho position must be unsubstituted.



V

Attempts by Keane and Nolan (*loc. cit.*) to effect dechlorination or fission of gangaleoidin with hydriodic acid gave unworkable products.

It will be noted from these earlier results that, with the exception of the position of the lactone bridge and the ether link, and a speculation as regards the position of the carboxylic ester group, no evidence was available regarding the positions of the various substituents in the benzene rings. Even the existence of two methyl substituents was inferred by analogy. In the present paper we have investigated further the constitution of gangaleoidin. A Kuhn-Roth determination established the presence of two C-methyl groups. Our experiments were next directed towards a re-examination of the dicarboxylic acid, referred to above, which was produced from gangaleoidin by the following series of reactions:—



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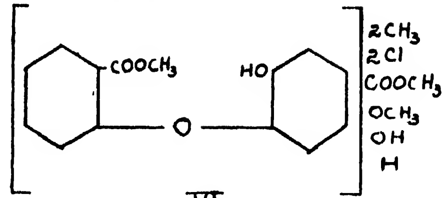

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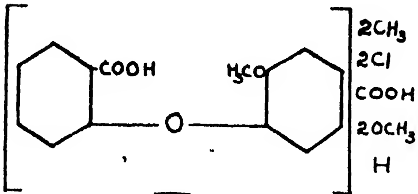

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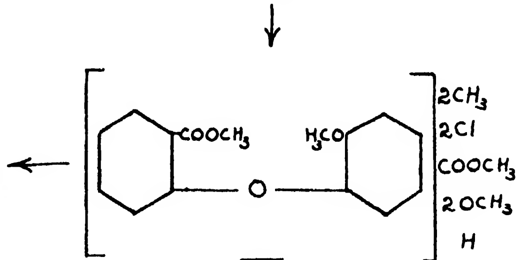
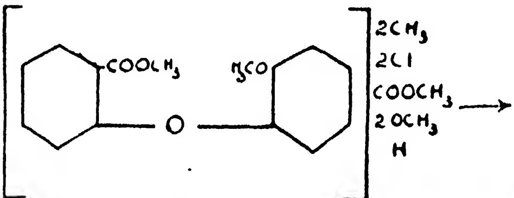
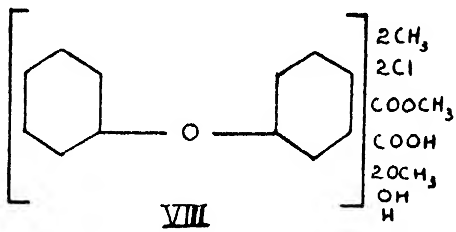


VII

The three different substances obtained by distilling in vacuo the product II are in accordance with the dicarboxylic acid structure attributed to the latter; there are, however, certain factors which justify one in entertaining the view that the hydrolysis of VII may have proceeded in accordance with the scheme

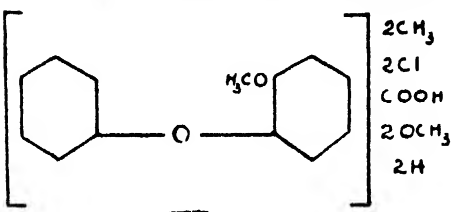


VII

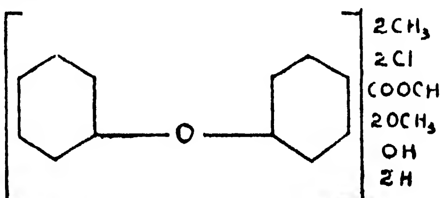


VIII

so that the product of hydrolysis instead of being a dicarboxylic acid may in fact be the phenol carboxylic acid carboxylic ester VIII. Thus, the dicarboxylic ester VI (*supra*), which contains free hydroxyl groups, on vigorous hydrolysis with alcoholic or aqueous potash loses only one methoxyl group (Keane and Nolan, *loc. cit.*). This would support the transformation VII → VIII. The easy demethylation of chlorinated phenol ethers is known (Calam and Oxford, J.C.S., 1939, p. 281). It would follow from the structures II and VIII that the product obtained by the loss of one molecule of carbon dioxide, which loss may be effected more readily by heating in anhydrous formic acid than by heating in vacuo, would have either of the formulae IX or X.

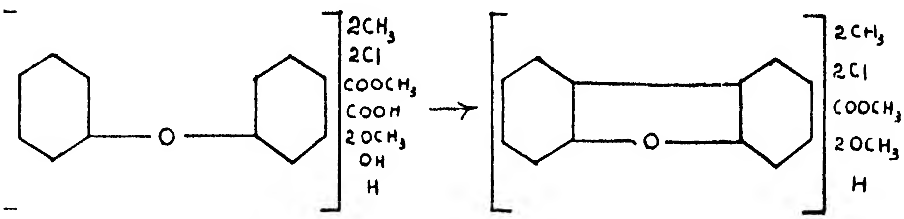


IX

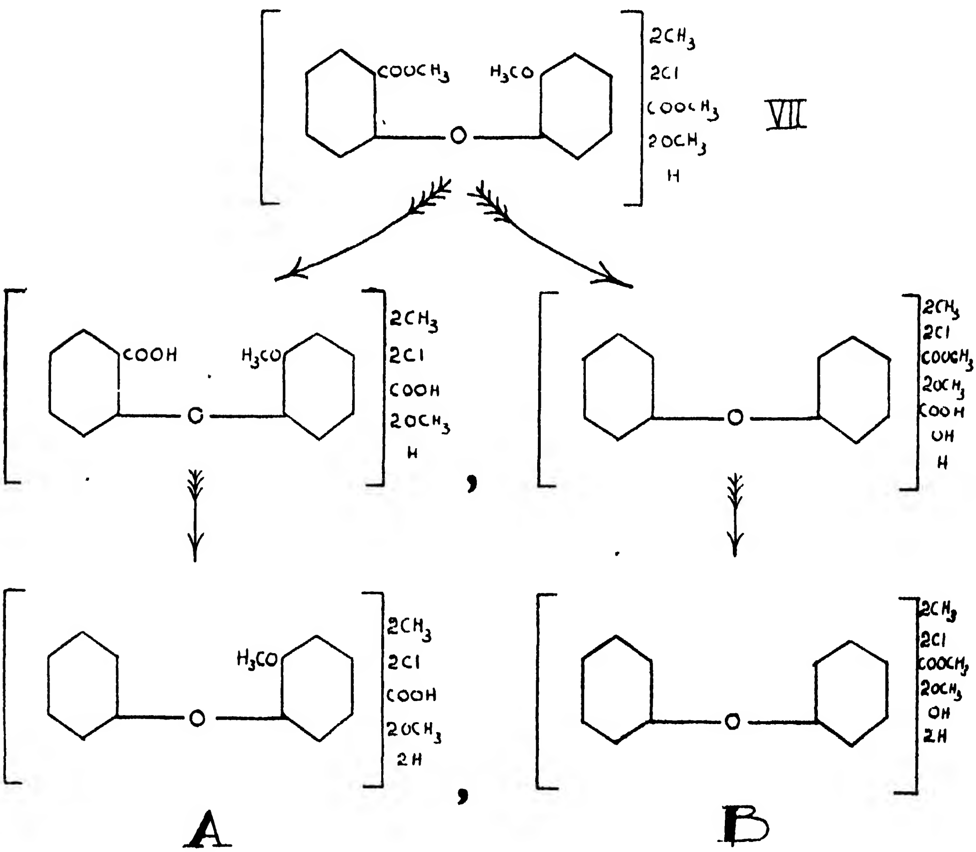


X

The insolubility of II (or VIII) in ligroin, and the solubility of the product obtained from it by decarboxylation, as well as the weak acidity of the latter, would favour the formula X for the latter. The only way, however, in which one could account for the loss of carbon dioxide and water from a substance of formula VIII would be a transformation of the type



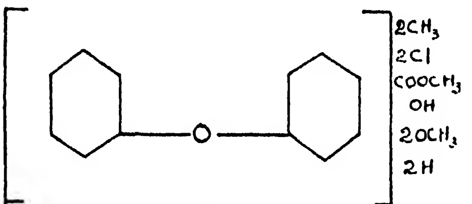
which would also explain the absence of salt formation with concentrated hydrochloric acid. It thus becomes a question of deciding whether the hydrolysis of VII followed by decarboxylation takes the course A or B.



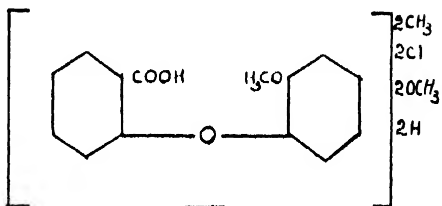
On treating the intermediate hydrolysis product with sulphuric acid and acetic anhydride the product is recovered unchanged. This favours the course A unless the hydroxyl group in the first stage of B is so acidic that it

does not readily acetylate. On the other hand, the final product obtained in the above series of reactions when treated with acetic anhydride and sulphuric acid does *not* acetylate, but loses a molecule of methyl alcohol, forming a lactone. This reaction is consistent with either of the courses A or B. However, if course B were correct, on treating the lactone with methyl alcoholic potash the original substance should be regenerated. In fact, a new isomer is obtained. Accordingly, the series of reactions is represented by course A, and hydrolysis of VII produces a dicarboxylic acid from which a monocarboxylic acid is produced by partial decarboxylation. It is peculiar that the dicarboxylic acid is unaffected by acetic anhydride and sulphuric acid, while the monocarboxylic acid forms a lactone. It is also worthy of note that methylation of the hydroxyl groups in gangaleoidin methyl ester facilitates the hydrolysis of *both* carboxylic ester groups, while in the case of the unmethylated product only *one* carboxylic ester group is hydrolysed.

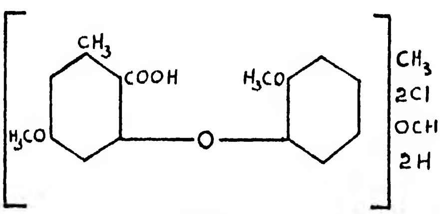
Whereas the monocarboxylic acid produced in the series of reactions A is readily methylated by diazomethane to an ester m.p. 78–79° C., the isometric compound which must have the structure XI



XI



XII



XIII

is not methylated under these conditions, nor is it readily extracted from ether solution with dilute sodium hydroxide. On the other hand, it is methylated in boiling acetone by dimethyl sulphate and potassium carbonate to the compound m.p. 78–79° C., referred to above.

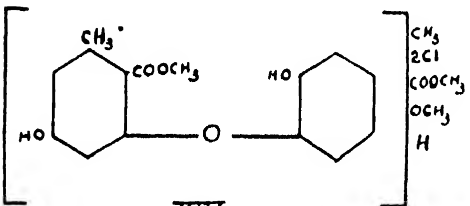
From the fact that the monocarboxylic acid undergoes ring closure with the elimination of methyl alcohol it must have the structure XII.

Further information with regard to its structure was obtained by treating its ester in carbon tetrachloride solution with chlorine followed by reduction with stannous chloride; 4·6 dichlor methyl orsellinate 5 methyl ether was isolated

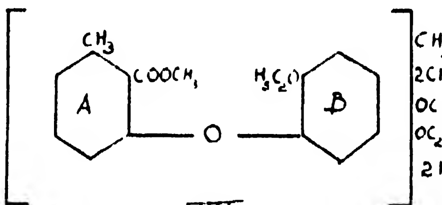
from the products of reaction. It thus follows that the monoacid has the structure XIII.

An attempt was next made to gain insight into the structure of gangaleoidin itself. The action of chlorine, followed by reduction with stannous chloride, on gangaleoidin methyl ester gave 4·6 dichlor methyl orsellinate. Gangaleoidin methyl ester must accordingly have the structure XIV.

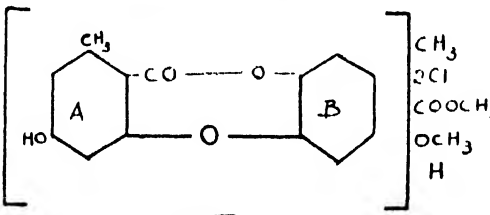
It is most unlikely that the series of reactions would have involved demethylation, as such did not occur in the case of similar experiments carried out to elucidate the structure of the monoacid. To confirm this, gangaleoidin methyl ester was ethylated with diazoethane, and by subsequent hydrolysis, decarboxylation, and methylation, the substance XV was formed. Chlorination followed by reduction yielded 4 6 dichlor methyl orsellinate 5-ethyl ether, thus confirming the presence of a hydroxyl group in ring A. Thus, gangaleoidin must have the structure XVI.



XIV



XV



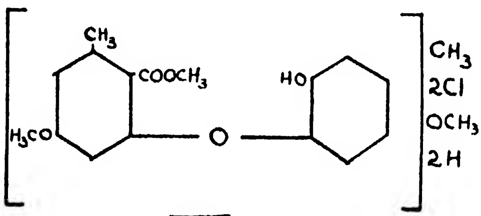
XVI

Obviously the second CH_3 group and the OCH_3 group must be in ring B. The establishing of the position of the hydroxyl group in ring A precludes the existence in gangaleoidin itself of a carboxylic ester group in ring A, because it would have to be ortho to the hydroxyl group, and such a system would give a positive reaction with ferric chloride. Gangaleoidin, however, does not give such a reaction, hence the ester group must be in ring B. Consequently there are in ring B methyl, methoxyl, and ester substituents, leaving only one possible position vacant, which must be occupied by either hydrogen or chlorine; thus there must be at least one chlorine atom in ring A.

With regard to the determination of the position of the ester group in ring B, a reaction using 2·6 dichlorquinone-chlorimide has been employed.

This reagent has been shown by Gibbs (J. Biol. Chem., 1927, **72**, p. 649), Theriault (Ind. & Eng. Chem., 1929, **21**, p. 343), and Todd (J.C.S., 1940, p. 1208) to give a blue colour in a sodium borate buffer (pH 9·2) with substances containing a hydroxyl group para to hydrogen, the reaction being essentially the formation of an indophenol. Our experiments indicate that the reaction also takes place if the position para to the hydroxyl is occupied by a COOH group or by a chlorine atom, but *not* if an ester group is present in the para position. The blue colour is modified by substituents, more particularly by the presence of other hydroxyl groups. The reaction resembles the known coupling of a diazonium salt with a p-hydroxy carboxylic acid with elimination of carbon dioxide.

Now gangaleoidin methyl ester XIV does not give an indophenol reaction, whereas the derivative XVII



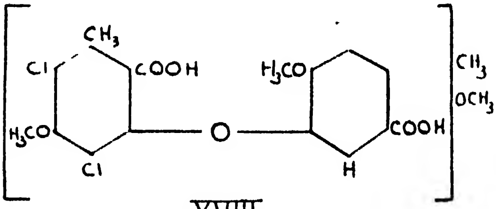
XVII

in which the ester group has been eliminated from ring B gives a bright blue colour. This is evidence that, in the case of gangaleoidin methyl ester, the ester group in ring B must be present in a position para to hydroxyl. Since the dicarboxylic acid II apparently forms a xanthone on distillation in vacuo, the position ortho to the oxygen bridge in ring B must contain hydrogen. Thus, in the case of gangaleoidin, there are in ring B, in addition to the lactone and oxygen bridges, methyl, methoxyl, ester, and hydrogen, so that the two chlorine atoms must be in ring A. This conclusion has been confirmed by a repetition of the thermal decomposition experiments of Keane and Nolan (Sci. Proc. R.D.S., **22**, 1940, p. 206) with gangaleoidin methyl ester, in which, in addition to the production of an isomer of gangaleoidin methyl ester, we also obtained a small amount of 4 6 dichlor orsellinic ester.

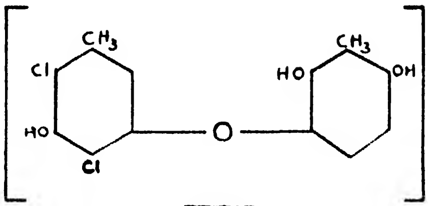
In order to fix the position of the methoxyl group in ring B gangaleoidin was demethylated in acetic acid solution, with hydrobromic acid in the presence of red phosphorus. The product obtained had the constitution $C_{14}H_{12}O_4Cl_2$, and on methylation gave the same product, m.p. 165° C., as was obtained by Keane and Nolan (*loc. cit.*) by the pyrolysis of the dicarboxylic acid II (*supra*), which acid we can now express by the formula XVIII.

The substance of constitution $C_{14}H_{12}O_4Cl_2$ gave a red coloration with bleaching powder, and accordingly must contain two hydroxyl groups meta to

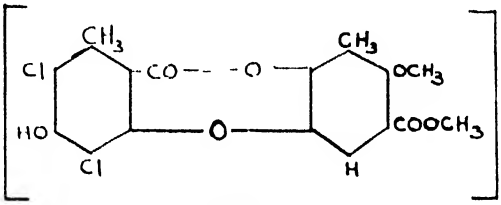
each other. Thus it must have the constitution **XIX**; so that **XX** must be the structure of gangaleoidin.



XVIII

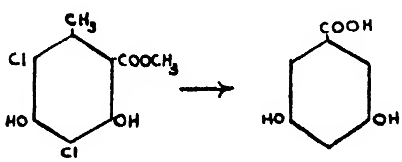


XIX



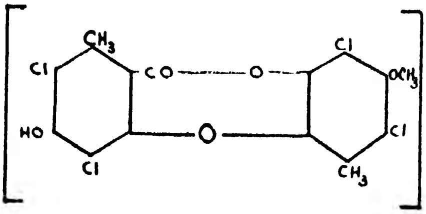
XX

It was thought that a potash fusion of gangaleoidin methyl ester should give confirmatory evidence of the orientation of the substituents in the B ring of gangaleoidin, as the replacement of the chlorine atoms of the A ring by hydroxyl during fusion should result in the eventual destruction of that ring, leaving the residue of the B ring. However, potash fusion of gangaleoidin methyl ester gave a product of formula $C_{11}H_{12}O_6$. This substance contains one carboxylic acid group, four hydroxyls, and probably one methyl group. It gives a blood-red coloration with bleaching powder, indicating the presence of hydroxyls meta to each other, and no ferric chloride reaction, showing that the carboxylic acid group is not ortho to hydroxyl. Owing to the large loss of material in the fusion it was not possible to investigate the constitution of the product in the desirable detail, but a potash fusion of 4 6 dichlor orsellinic ester gave a possible clue to its structure. It has been found that dichlor orsellinic ester on potash fusion gives α-resorcylic acid

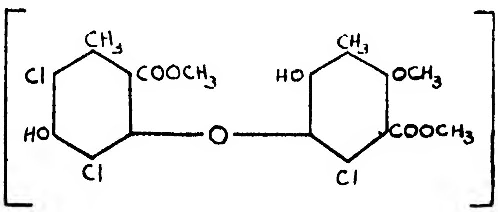


the chlorine atoms not being replaced by hydroxyls in the process as would be expected. Incidentally, a good yield of α-resorcylic acid was obtained by a

potash fusion of diploicin, which one of us (T.J.N.) believes to have the constitution



Thus gangaleoidin in the process of potash fusion apparently undergoes an intramolecular rearrangement accompanied by oxidation, resulting in the production of a tetrahydroxy diphenyl carboxylic acid derivative, made up of the two units, α-resorcylic acid and 2·6 dihydroxytoluene. Any question of the possible existence in gangaleoidin of a diphenyl structure with a non-reactive hydroxyl group is, apart from other considerations, excluded by the fact that gangaleoidin methyl ester can be chlorinated to a monochlor derivative in which the chlorine has entered the B ring. This derivative, on the above considerations, would have the constitution



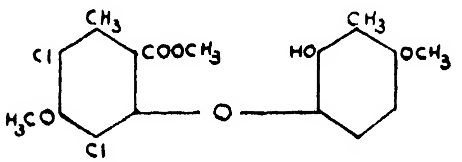
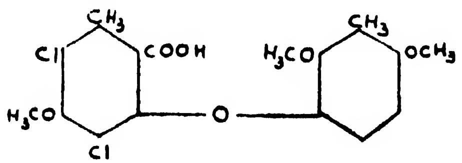
It can be converted to a dimethyl ether, which, in turn, can be hydrolysed to a dicarboxylic acid which resists even partial decarboxylation owing to the steric effect of the ortho chlorine atom.

EXPERIMENTAL.

Reactions of 2 : 6 Dichlorquinone Chlorimide with various Oxy-compounds.

2 : 6 dichlorquinone chlorimide was prepared according to the method of Kollrepp (A. 234·18). The reagent was used in the presence of a sodium borate buffer (Palitzsch, J. Biol. Chem., 1927, **72**, 651) A small quantity of the substance under test was taken in a test tube and to it was added about 10 c.c. of the solution of the sodium borate buffer (pH 9 2), a small amount of alcohol being added in the case of substances soluble with great difficulty; there

was then added a drop or two of a suspension of the quinonechlorimide in water. The colour developed rapidly in most cases.

Substance.	Colour.	Reaction.
Phenol	Pure Blue	+
Catechol	Bluish purple	+
Resorcinol	Violet red	+
Orcinol	Reddish Purple	+
p-chlorphenol	Intense Blue	+
p-hydroxy Benzoic acid	Deep blue	+
p-hydroxy Benzoic ester	Olive brown	—
Gangaleoidin	Olive brown	—
Gangaleoidin Methyl ester	Steel colour	—
Gangaleoidin chlor-methyl ester	Slate grey	—
Diploicin	Olive brown	—
Diploicin Methyl ester	Intense blue	+
4 6 dichlor Methyl orsellinate	Violet purple	+
	Blue	+
	Olive brown	—

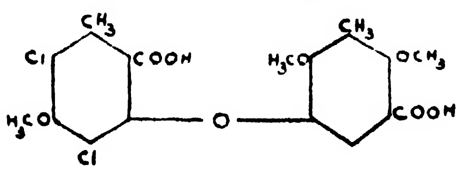
Kuhn-Roth Estimation of C-Methyl in Gangaleoidin.

7 520 mg. ... ... 4 25 c.c. N/100 NaOH.

Found C-Methyl = ·8·5 %.

$(CH_3)_2C_{16}H_8O_7Cl_2$ requires C-Methyl = 7·2 %.

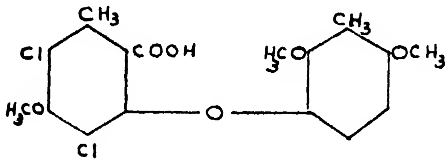
Attempted Acetylation of the Dicarboxylic Acid.



0 05 g. of dicarboxylic acid, m.p. 216–217° C., was dissolved in 3 c.c. acetic anhydride, and a few drops of sulphuric acid added. The light straw-coloured solution was allowed to stand for two hours. It was then poured into 10 c.c.

water, and after standing a further two hours the precipitate was filtered off and found to be the original compound unchanged.

Action of Acetic Anhydride and Sulphuric Acid on the Monocarboxylic Acid.

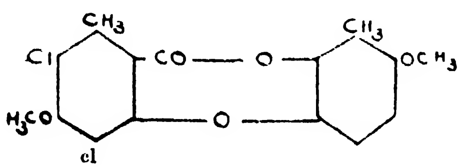


0·1 g. of monocarboxylic acid, m.p. 138–139° C., was dissolved in 6 c.c. acetic anhyride, a few drops of sulphuric acid were added, and the whole allowed to stand for two hours. It was then poured into 20 c.c. water, and after two hours the precipitate collected and crystallized from 20 c.c. hot ligroin. A substance, m.p. 208–209° C., was obtained.

4·355 mg. dried at 100° C in vac. No loss.
4·355 mg. gave 8 850 mg. CO_2; 1 420 mg. H_2O.
5 457 mg. gave 4·160 mg. AgCl.
3 890 mg. gave 5·280 mg. AgI.

Found: C = 55·4; H = 3·62; Cl. = 18 7; OCH_3 = 17·8.
$C_{15}H_8O_3Cl_2(OCH_3)_2$ requires C = 55·28; H = 3·79; Cl = 19·2; OCH_3 = 16·8.

Action of Methyl Alcoholic Potash on Compound.



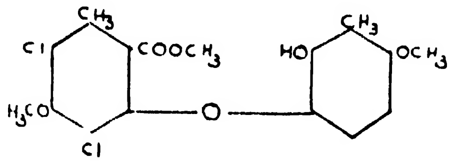
0·05 g. of compound, m.p. 208–209° C., suspended in 1 c.c. methyl alcohol was heated under a reflux with 5 c.c. of 5 % methyl alcoholic potash for one hour. On cooling, the solution was filtered and acidified. The white precipitate was collected and crystallized from ligroin and gave a material, m.p. 164° C.

No loss in weight when dried in vacuo at 100° C.
3·353 mg. gave 6·650 mg. CO_2 and 1·350 mg. H_2O.
5·137 mg. gave 4·060 mg. AgCl.
5·048 mg. gave 8·510 mg. AgI.

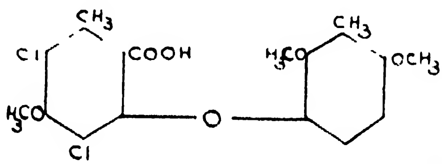
Found: C = 54·09; H = 4·4; Cl = 19·5; OCH_3 = 22·2.
$C_{15}H_9O_3Cl_2(OCH_3)_3$ requires C = 53·9; H = 4·5; Cl = 17·7; OCH_3 = 23·2.

The product gives no colour with either ferric chloride or bleaching powder, but it gives a bright blue indophenol reaction.

Methylation of Compound.

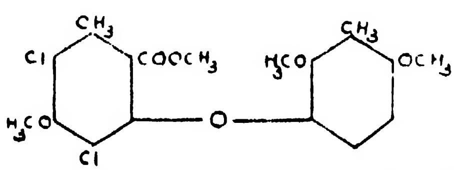


The substance, m.p. 164° C., did not methylate readily with diazomethane. 0 3 g. of the compound was dissolved in 30 c.c. dry acetone and boiled for 1½ hours with 2 c.c. dimethyl sulphate and 4 g. anhydrous potassium carbonate. Then 0 5 c.c. dimethyl sulphate and 2 g. potassium carbonate were added, and heating continued for a further hour. On cooling, the solution was filtered from the potassium carbonate and allowed to evaporate to dryness, with a final drying over potassium hydroxide in a vacuum desiccator. The residue was then taken up in ether, and extracted with a 1 per cent. solution of sodium hydroxide. The ether layer was washed with acid and finally with water before drying. The ether was evaporated off, and steam blown through the residual oil to remove the excess dimethyl sulphate. The residue was taken up in ether, washed, dried, and the solvent evaporated leaving a light yellow gum, which crystallized readily from methyl alcohol to give a substance, m.p. 78–79° C. On admixture with the compound formed by direct action of diazomethane on the mono-carboxylic acid,



there was no depression in melting point. The ester gives no reaction with either bleaching powder, ferric chloride, or 2 : 6 dichlor quinone chlorimide.

Action of Chlorine followed by Reduction on Compound.



0 08 g. of the compound, m.p. 78–79° C., was dissolved in 10 c.c. chloroform, and to the solution was added 5 c.c. of a solution of chlorine in carbon tetrachloride containing 0 04 g. Cl per c.c. After standing overnight the solution was poured into a beaker and evaporated, leaving a brown gum which did not crystallise from methyl alcohol, ligroin, or 40/60 petrol. The

gum was dissolved in 3 c.c. acetic acid, and to this was added a solution of 0 3 g. stannous chloride in 3 c.c. of 20 per cent. hydrochloric acid. The oil lightened in colour to yellow, and on dilution with water a yellow oil was precipitated (A). On further dilution of the decanted mother liquors and long standing, a small amount of white needles separated, which had a melting point less than 100° C. These crystals were soluble in sodium hydroxide without colour change; they gave a violet colour with ferric chloride, and no reaction with bleaching powder.

The oil (A) was dissolved in 5 c.c. of 10 per cent. methyl alcoholic potash; the solution after filtration was heated for 1 hour in a hydrogen atmosphere, after which time it was diluted with twice its volume of water, and acidified. The precipitate was taken up in ether, and extracted with sodium bicarbonate, which gave a small amount of crystals giving a port colour with ferric chloride. The ethereal solution was then extracted with 1 per cent. sodium hydroxide, yielding a substance which, after washing with ligroin, was crystallized from dilute methyl alcohol, and gave a compound, m.p. 77–77 5° C. This compound gave a purple ferric chloride reaction, no bleaching powder reaction, and a violet purple indophenol reaction.

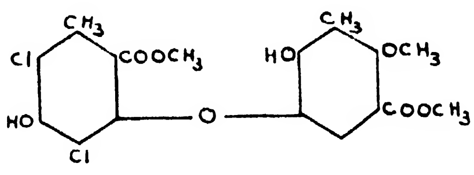
3 657 mg. gave 6 100 mg. CO_2; 1·230 mg. H_2O.
3 569 mg. gave 6 200 mg. AGI.
3 670 mg. gave 3 900 mg. AgCl.
0 632 mg. in 6 368 mg. camphor gave D 14 8°.

Found: C = 45·49; H = 3·74; Cl = 26 3; OCH_3 = 22·9.
$C_{10}H_{10}O_4Cl_2$ requires C = 45 28; H = 3 77; Cl = 26 8; OCH_3 = 23 4.

Found: M.W. = 268 required = 265.

On admixture with 5 methoxy 4 6 dichlor o-orsellinic methyl ester, m.p. 79–80° C., the mixed melting point showed no depression.

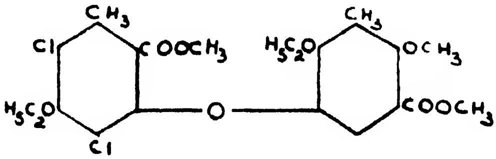
Ethylation of Gangaleoidin Methyl Ester.



0·4 g. of gangaleoidin methyl ester in 20 c.c. acetone was ethylated with an ethereal solution of diazoethane (from 8 c.c. nitroso-n-ethyl urethane). After standing overnight the solvents were evaporated, and the residue crystallized from hot ligroin. Crystals were obtained, m.p. 155° C.

4·542 mg. gave 10·670 mg. AgI = 31·0% OCH_3.
$C_{16}H_7O_3Cl_2(OCH_3)_3(OC_2H_5)_2$ requires 30·9% OCH_3.

Hydrolysis of the Ethylated Compound.

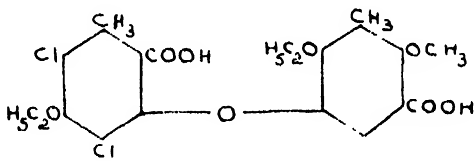


0·8 g. of compound was heated for 14 hours under reflux with 80 c.c. of 15 per cent. methyl alcoholic potash, after which time a sample on dilution with water showed no turbidity. After evaporating off the alcohol in an air draught the residue was diluted with 200 c.c. water, and acidified. The precipitate, after standing overnight, was collected and crystallized from 50 per cent. aqueous alcohol, and gave a substance, m.p. 215° C.

4·100 mg. gave 8·100 mg. CO_2 and 1·710 mg. H_2O.
4·907 mg. gave 3·170 mg. AgCl.
4·082 mg. gave 6·420 mg. AgI.

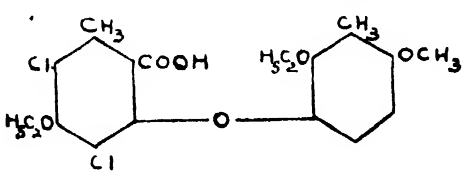
Found: C = 53·88; H = 4·6; Cl = 16·0; OCH_3 = 20·8.
$C_{14}H_7OCl_2(COOH)_2(OCH_3)(OC_2H_5)_2$ requires C = 53·3; H = 4·65; Cl = 15·0; OCH_3 = 19·6.

Decarboxylation with Formic Acid of Compound.



1·0 g. was heated to boiling under reflux with 10 c.c. 98–100 per cent. formic acid for half an hour. After cooling, the solution was diluted with 40 c.c. water, and the precipitate allowed to stand overnight. It was collected, and gave a product, m.p. 161–163° C.

Methylation of Monocarboxylic Acid.



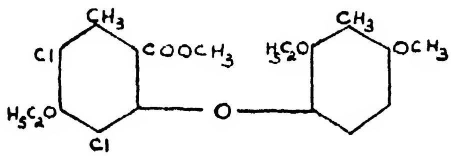
0·5 g. of the compound, m.p. 161–163° C., in 10 c.c. dry acetone was methylated with an ethereal solution of diazomethane (from 15 c.c. nitroso-N-methyl urethane), and allowed to stand overnight. On evaporation of the

solvents the residue was crystallized from warm methyl alcohol yielding a substance, m.p. 103–104° C.

3·623 mg. gave 7·480 mg. CO_2, 1·630 mg. H_2O.
5·132 mg. gave 3 360 mg. AgCl.
4·965 mg. gave 10 720 mg. AgI.

Found: C = 56·30; H = 5 0; Cl = 16 2; OCH_3 = 28 5.
$C_{14}H_8OCl_2(COOCH_3)(OC_2H_5)_2(OCH_3)$ requires C = 56·88; H = 5·46; Cl = 16 0; OCH_3 = 28.

Action of Chlorine followed by Reduction on the Compound.



To a solution of 0·6 g. of compound, m.p. 103–104° C., in chloroform was added 13 3 c.c. of chlorine in carbon tetrachloride which contained 0·04 g. chlorine per c.c. After standing overnight the excess chlorine was removed by blowing dry air through the solution, and the solvents removed by evaporation. The residue was a greenish oil. This oil was reduced in acetic acid solution (30 c.c.) with 2 g. stannous chloride in 60 c.c. of 20 per cent. hydrochloric acid. The colour lightened, and on standing an oily material was precipitated. The mother liquors were decanted, and the solid washed with a little 5 per cent. hydrochloric acid and then with water. The oil was then boiled with 30 c.c. of 10 per cent. methyl alcoholic potash under reflux for one hour. After filtering off sodium chloride and diluting with twice its own volume of water the solution was acidified, and the precipitate extracted with ether. The ethereal solution was extracted with 3 per cent. sodium bicarbonate and then 1 per cent. sodium hydroxide. After acidifying the hydroxide layer, the white precipitate was taken up in ether, washed, dried, and the ether evaporated. The solid recovered was crystallized from dilute methyl alcohol, and a substance, m.p. 88–89° C., was obtained.

3 910 mg. gave 6·780 mg. CO_2 and 1·570 mg. H_2O.
4·958 mg. gave 5·160 mg. AgCl.
4 853 mg. gave 7 940 mg. AgI.

Found: C = 47·3; H = 4 46; Cl = 25·7; OCH_3 = 21·6.
$C_7H_4OCl_2(OC_2H_5)(COOCH_3)$ requires C = 47·3; H = 4·3; Cl = 25·4; OCH_3 = 22 2.

Action of Chlorine followed by Reduction on Gangaleoidin Methyl Ester.

7 g. of the compound were dissolved in 200 c.c. chloroform, and to the solution was added 115 c.c. of a solution of chlorine in carbon tetrachloride (1 c.c. of solution contained 0·04 g. chlorine per c.c.), the whole, being tightly stoppered, was left overnight. Air was bubbled through the solution to remove the excess chlorine, and then the solvents were distilled off under reduced pressure, leaving a dark brown gum. The gum was reduced in acetic acid solution (175 c.c.) by 12 g. stannous chloride in 360 c.c. of 20 per cent. hydrochloric acid. The colour lightened to yellow and solid separated on dilution.

After standing overnight the liquors were decanted from a gum. The gum was taken up in ether, and extracted successively with 3 per cent. sodium bicarbonate, 3 per cent. sodium carbonate, and 3 per cent. sodium hydroxide. The decantate was extracted with chloroform, and the extract recovered from the chloroform. On distillation with superheated steam, the chloroform extract, the sodium bicarbonate, and sodium hydroxide extracts all yielded the same substance, m.p. 114–115° C., which on admixture with 4 6 dichlor o-orsellinic methyl ester showed no depression in melting point. The sodium carbonate extract was recovered and washed with cold benzene, leaving behind a substance, m.p 114–115° C., which was 4 6 dichlor o-orsellinic methyl ester.

4 608 mg. gave 4·260 mg. AgI.
2 582 mg. gave 2·820 mg. AgCl.

Found: OCH_3 = 12 15; Cl = 27 0.
$C_7H_5O_2Cl_2(COOCH_3) . \frac{1}{2}H_2O$ requires OCH_3 = 11 9; Cl = 27 3.

The benzene solution on evaporation gave a residue which on crystallization from benzene gave crystals, m.p. 156° C., which was the monochlor derivative of gangaleoidin methyl ester.

4 007 mg. gave 6 890 mg. CO_2 and 1 300 mg. H_2O.
4 790 mg. gave 4·430 mg. AgCl.
4 775 mg. gave 7 200 mg. AgI.

Found: C = 46 9; H = 3·6; Cl = 22 9; - OCH_3 = 19 9.
$C_{16}H_8O_5Cl_3(OCH_3)_3$ requires C = 47 5; H = 3·5; Cl = 22·2; - OCH_3 = 19 4.

The compound gives a negative ferric chloride reaction, a yellow colour with bleaching powder, and a slate-grey colour with 2 : 6 dichlor quinone chlorimide.

Methylation of the Monochlor-derivative of Gangaleoidin Methyl Ester, m.p. 156° C.

1·0 g. of the compound in acetone solution was methylated with an ethereal solution of diazomethane (10 c.c. nitroso-N-methyl urethane). After standing

for 22 hours the solvents were evaporated to a greenish oil, which crystallized from methyl alcohol, m.p. 98–100° C.

5 948 mg. gave 13 820 mg. AgI.

Found: OCH_3 = 30·6.
$C_{16}H_6O_3Cl_3(OCH_3)_5$ requires — 30 5.

0 9 g. of the compound, m.p. 98–100° C., was boiled for 5 hours with 63 c.c. of 15 per cent. methyl alcoholic potash. The solution was evaporated to dryness in air. The solid residue was dissolved on 45 c.c. water, acidified to 5 per cent. alkalinity, and filtered. The filtrate was then made acid, and extracted with ether. A brown gum was obtained on evaporating the solvent, which crystallized from aqueous methyl alcohol, and gave a product, m.p. 227–228° C.

3 931 mg. gave 6 620 mg. CO_2 and 1 410 mg. H_2O.
4 718 mg. gave 4 000 mg. AgCl.
4 098 mg. gave 5 750 mg. AgI.

Found: C = 45 9; H = 3 98; Cl = 21 1; OCH_3 = 18 5.
$C_{16}H_8O_5Cl_3(OCH_3)_3 . H_2O$ requires C = 45 8; H = 3 8; Cl = 21 4; OCH_3 = 18 7.

Attempted Decarboxylation of the above Dicarboxylic Acid, m.p. 227–8° C.

0 44 g. compound was boiled with 15 c c. of 98 per cent formic acid for 30 minutes. The solution was then cooled and diluted. After standing for two hours the precipitate was collected, m.p. 222° C. It was the unchanged dicarboxylic acid, and was methylated back to the compound, m.p. 98–100° C., referred to above, with diazomethane.

Vacuum Distillation of Gangaleoidin Methyl Ester, m.p. 197° C.

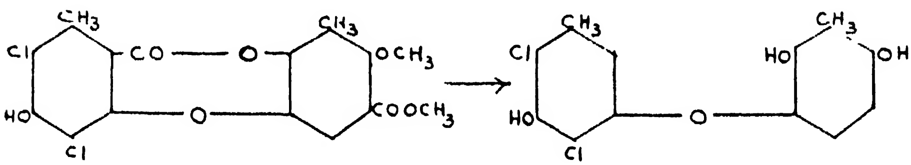
2 g. were distilled under reduced pressure in four portions of 0·5 g. each. The distillate, a red oil, was washed with 8 c.c. warm ligroin, and the solvent decanted. To the decantate was added 2 c.c. benzene, and the solution left overnight, when it was again decanted from some oily solid. The residue, obtained by evaporating the decantate, was crystallized from 50 per cent. aqueous methyl alcohol, and had a melting point 112–113° C., which was not depressed on admixture with a sample of 4 : 6 dichlor o-orsellinic methyl ester. The red oil insoluble in warm ligroin was dissolved in 4 c.c. benzene, and after some oil had separated on standing, the solution was decanted. After some time a white globular solid was precipitated, which was washed with a few drops of alcohol. The product was finally crystallized from a mixture of 1 c.c. benzene and 1 c.c. ligroin, and yielded a product, m.p. 180–181° C., which on admixture with the original material melted at 166° C. The compound gave a

blue quinone chlorimide reaction, whereas the original material yields a steel colour.

4·717 mg. gave 8·825 mg. CO_2; 1 690 mg. H_2O.
3·488 mg. gave 5·330 mg. AgI.
8·669 mg. gave 5 490 mg. AgCl.
0 281 mg. in 4·007 mg. camphor gave 5·6° D.
0 270 mg. in 6 059 mg. camphor gave 3·5° D.

Found: C — 51 03; H = 3 98; Cl = 15·7; OCH_3 = 20 2; MW = 501; 509.
$C_{16}H_9O_5Cl_2(OCH_3)_3$ requires C = 51·24; H = 4 04; Cl = 16 0; OCH_3 = 20·9; MW = 445.

Demethylation of Gangaleoidin.



0 5 g. gangaleoidin in 10 c.c. glacial acetic acid was heated with 1 g. red phosphorus and 8 c.c. constant boiling hydrobromic acid (b.p. 126° C; 47 8 per cent.) for six hours under a reflux. After cooling, the whole was diluted with 100 c.c. of water and saturated with sodium bisulphite to decolourize. When the mixture had stood overnight it was filtered. From the filtrate was recovered by ether extraction a product which was a yellow oil and showed no inclination to crystallize. During treatment with steam a change appeared to occur as the product gradually went into solution in the hot water, from which it crystallized in small granular plates, m.p. 111–112° C. It was soluble in 3 per cent. sodium carbonate solution, and contained halogen. The compound gave no colour with ferric chloride, a blood-red colour with bleaching powder, and a dark pink with 2: 6 dichlor quinone chlorimide.

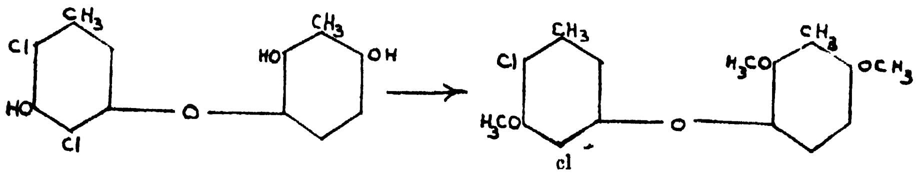
4 278 mg. dried at 50° C. showed no loss.

When the compound is dried at 100° C. in vacuo complete sublimation occurs.

4·278 mg. gave 7·900 mg. CO_2; 1·670 mg. H_2O.
5 030 mg. gave 4 230 mg. AgCl.
Methoxyl negative.

Found: C = 50·36; H = 4·3; Cl = 20·8.
$C_{14}H_{12}O_4Cl_2 . H_2O$ requires C = 50·4; H = 4·2; Cl = 21·3.

Methylation of Product Obtained in the Demethylation of Gangaleoidin.



0·1 g. of the demethylated compound was dissolved in 5 c.c. of 3·5 N caustic soda, and shaken with 1·5 c.c. dimethyl sulphate for 30 minutes, after which time a solid separated. The reaction was finished by heating the whole on a boiling water bath. The solid was collected, crystallized from 60/80 petrol ether, and washed with methyl alcohol, when a product, m.p. 108–109° C. was obtained. A mixed melt with the methyl ether of the phenolic body obtained by heating the dicarboxylic acid, m.p. 216° C., in glycerine (Nolan and Keane, *loc. cit.*) showed no depression.

3·901 mg. gave 8·240 mg. CO_2; 1·800 mg. H_2O.
4·264 mg. gave 8·140 mg. AgI.

Found: C = 57·6; H = 5·12; OCH_3 = 25·2.
$C_{14}H_9OCl_2(OCH_3)_3$ requires C = 57·14; H = 5·04; OCH_3 = 26·1.

Acidification of the alkaline liquors from this methylation gave a solid which on crystallization from aqueous methyl alcohol had m.p. 165–166° C., and was identical with the phenolic body obtained by heating the dicarboxylic acid, m.p. 216° C., in glycerine (Nolan and Keane, *loc. cit.*). The product gives a blue indophenol reaction identical with that obtained with the phenolic body; neither the product obtained here nor the phenolic body gave a colour with bleaching powder or ferric chloride.

Potash Fusion of Gangaleoidin Methyl Ester.

20 g. potassium hydroxide pellets and 2 c.c. water were warmed to 100° C. in a nickel crucible. (A thermometer with the bulb encased in a brass sleeve served to read the temperature and acted as a stirrer.) 1 g. gangaleoidin methyl ester was added to the melt, and the temperature was raised gradually to 250° C., where it was maintained for 20 minutes. A further period of 5 minutes at 310° C. completed the fusion. The mass was then allowed to cool, was dissolved in 100 c.c. water, and the solution saturated with carbon dioxide. After acidification with dilute sulphuric acid the solution was extracted with ether. The brown gum obtained on evaporating the solvent was boiled in water with norite, and filtered. From the solution, large colourless crystals separated, which decomposed at 263° C. with previous browning. The product gave a blood-red bleaching powder reaction, but no coloration with ferric chloride. It contains no methoxyl.

C–CH_3 determinations.

12 220 mg. – 2·58 c.c. N/100 NaOH.
8 128 mg. – 1 10 c.c. N/100 NaOH.
3 637 mg. gave 7 270 mg. CO_2 and 1 690 mg. H_2O.
3·502 mg. in vacuo @ 110° C. lost 0·315 mg.
2·988 mg. in vacuo @ 110° C. lost 0·260 mg.
4 005 mg. in vacuo @ 105° C. lost 0 405 mg.

Found: C = 54 5; H = 5·1; C–CH_3 = 3·16, 2 02; H_2O = 8 99, 8 72, 10·1.
$C_{14}H_{12}O_6 1\frac{3}{4}H_2O$ requires C = 54 63; H = 5 04; C–CH_3 = 4 88; H_2O = 10·4.

3 565 mg. (vac. dried) gave 7 900 mg. CO_2 and 1 490 mg. H_2O.

Found: C = 60 4; H = 4 3.
$C_{14}H_{12}O_6$ requires C = 60 87; H = 4 35.

Acetylation of the Fusion Product.

0 05 g. of the compound was dissolved in 3 c.c. acetic anhydride and 1 drop of sulphuric acid, and allowed to stand for two hours. The solution was then poured on to 10 g. crushed ice, and allowed to stand overnight, when crystals, m.p. 178–179° C., separated, which were crystallized from benzene The crystals were easily soluble in sodium carbonate.

3·598 mg. gave 7 780 mg. CO_2 and 1 410 mg. H_2O.
3 908 mg. gave 8 500 mg. CO_2 and 1 610 mg. H_2O.
7·881 mg. – 7·19 c.c. N/100 NaOH.
0 627 mg. in 5·723 camphor gave 10 2 D.

Found: C = 59 1; 59 3. H = 4 3; 4 5. CH_3CO = 39 2. M.W. = 398.
$C_{14}H_8O_6(CH_3CO)_4$ requires C = 59·46; H = 4·55; CH_3CO = 38 74; M.W. = 444.

Methylation of the above Acetyl Derivative.

0 1 g. of the acetyl derivative was methylated in acetone solution with an ethereal solution of diazomethane (4 c.c. nitroso-N-methyl urethane). After 20 hours the solvents were evaporated, and the solid crystallized from methyl alcohol, m.p. 109–110° C.

3·584 mg. gave 7 920 mg. CO_2 and 1·570 mg. H_2O.
3 779 mg. gave 8 210 mg. CO_2 and 1·700 mg. H_2O.
3 690 mg. gave 2 050 mg. AgI.
4 110 mg. gave 2 240 mg. AgI.
8·020 mg. – 7 36 c.c. N/100 NaOH.

Found: C = 60 27; 59·60. H = 4 87; 5·03. OCH_3 = 7·37; 7·25. CH_3CO = 39 4.
$C_{14}H_7O_5(OCH_3)(CH_3CO)_4$ requires C = 60·26; H = 4·8; OCH_3 = 6 77; CH_3CO = 37·55.

Methylation of the Fusion Product.

0 1 g. of the compound was dissolved in 2 2 c.c. of 2N . NaOH. To this solution were added three separate lots of 0·12 c.c. dimethyl sulphate, shaking vigorously after each addition. Finally, 0·6 c.c. of 2 N . NaOH was added, and the whole heated under a reflux condenser on a boiling water bath for half an hour. After acidification the ether extract was evaporated to a solid, which crystallized from methyl alcohol, m.p. 178° C.

3 580 mg. gave 8 570 mg. CO_2 and 2 000 mg. H_2O.
3·784 mg. gave 10 040 mg. AgI.
0·737 mg in 6 670 mg camphor gave 11 8° D.

Found: C = 65 2; H = 5 9; OCH_3 = 35 0; M.W. = 346.
$C_{14}H_6O_2(OCH_3)_4$ requires C = 65 06; H = 6 02; OCH_3 = 37 35; M.W. = 332.

Potash Fusion of 1 6 Dichlor O-Orsellinic Methyl Ester, m.p. 113° C.

20 g. potassium hydroxide pellets and 2 c.c. water were warmed to 100° C. in a nickel crucible. 0·9 g compound was added to the melt, and the temperature was raised gradually to 250° C., where it was maintained for 20 minutes. A further period of 5 minutes at 310° C. completed the fusion. The mass was then allowed to cool, was dissolved in 100 c.c water, and the solution saturated with carbon dioxide. After acidification with dilute sulphuric acid the solution was extracted with ether. The brown gum obtained on evaporating the solvent was boiled in water with norite, and filtered. A white solid was obtained on concentrating the solution, which was crystallized from a mixture of acetic acid and benzene (1 : 2), m.p. 229–230° C. The product on admixture with a sample of α-resorcylic acid showed no depression in melting point.

Potash Fusion of Diploicin.

20 g. potassium hydroxide pellets and 2 c.c. water were warmed to 100° C. in a nickel crucible and 1 g. of diploicin was added. The temperature was carefully raised to 250° C., where it was maintained for 20 minutes. A further period of five minutes at 310° C. completed the fusion. When the melt had cooled, it was dissolved in 100 c.c. water, the solution saturated with carbon dioxide, acidified with dilute sulphuric acid, and extracted with ether. The ethereal solution on evaporation gave a good yield of oily crystals, which were clarified by boiling in aqueous solution with norite. The solid obtained on concentrating the aqueous liquors was crystallized from a mixture of acetic acid and benzene (1 : 2), yielding a product, m.p. 228–229° C., not depressed on admixture with α-resorcylic acid.

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ASCORBIC ACID.

PART 3: THE ASCORBIC ACID CONTENT OF FRUITS AND VEGETABLES GROWN IN EIRE.

BY EINHART KAWERAU.

Price One Shilling and Sixpence.

No. 18.

ASCORBIC ACID.

PART 3: THE ASCORBIC ACID CONTENT OF FRUITS AND VEGETABLES GROWN IN EIRE.

By EINHART KAWERAU.

[Read November 23, 1943. Published separately January 28, 1944.]

In April, 1940, Fixsen and Roscoe published a series of tables of the vitamin content of human and animal foods. The figures very largely represent values obtained by estimations carried out by the authors themselves, as well as a selection of results chosen from over two hundred references to the world literature. Figures from Ireland are not included in this work of reference, because no data have hitherto been available. The work here presented was designed to fill this gap, and at the same time it was intended to show from what part of the dietary the Irish population received its main supply of vitamin C, in order that during emergency conditions this main supply might be adequately protected.

In connection with the task of estimating ascorbic acid in foods, two complex problems commonly arise. One concerns the methods of detecting and estimating the vitamin; the other the stability of the vitamin in its natural and in artificial environment. Earlier communications to this Society deal with these two problems, respectively (Fearon and Kawerau, 1943, 1944).

It has been already stated that the purpose of this investigation is twofold, and the following will serve to illuminate the issue more clearly. Vitamin C is produced during the growth period of the plant, and one's enquiry may be directed to estimate correctly the number of factors that determine the optimum production of the vitamin in a particular plant. This research, if it covers not one but a number of species, will supply answers to the following questions:—(1) What plants in this country contain the vitamin in the highest concentration? (2) At what time of the year is the highest concentration reached? (3) Does this period coincide with the best time for harvesting a particular plant? (4) Do the customary methods of tilling and soil treatment in this country influence the production of the vitamin favourably or adversely? All these questions relate to one type of enquiry; a second quite distinct line of enquiry would comprise the following questions:—Vitamin C is taken at every meal. Taking all quantitative and qualitative considerations into view, which is the main dietary source of the vitamin of the nation? What is the degree of destruction of the vitamin during transport and distribution to the

town consumer? How is it affected by home and commercial food processing? And, finally, what is the cheapest dietary source of the daily adult human requirements of the vitamin?

The limited facilities at our disposal have made it impossible to answer all the questions just set out. Where personal observations are lacking, and where it is permissible, reference will be made to results obtained by other workers in the same field.

Method of Enquiry.

The principles of representative sampling, as stated by Harris and Olliver (1942), were strictly adhered to where information concerning optimum plant production of the vitamin was desired. These principles include:—(*a*) if different plants are investigated, they should be grown on the same piece of ground; (*b*) if fruit at different stages of ripeness is selected, it should be picked from the same plant, or better still, from the same branch; and (*c*) a minimum of time should elapse between picking the plant, and carrying out the estimation. None of these principles need be observed when the dietary source of the vitamin is the chief concern. Random samples were selected from a number of shops (in Dublin), and the results were pooled. Where different methods of processing of food are studied, it is important to do a sufficiently large number of estimations in order to obtain significant average results characteristic of the particular process adopted.

Method of Analysis.

The considerations given in our first report (Fearon and Kawerau, 1, 1943) and the desire to make the results comparable with those of most other workers led me to choose titration with 2:6-dichlorophenol-indophenol, as the method used in this particular work. The procedure is as follows:—One side of a balance is modified so as to hold in suspension a 2 oz. mortar. The mortar is filled with 20 ml. of strong (16 per cent.) metaphosphoric acid that has been kept on ice. The balance is brought to equilibrium, and from 5 to 20 g. of the substance to be analysed cut with a horn spatula directly into the acid (if cutting is required). The amount taken is weighed, the mortar is removed from the balance, and the substance is thoroughly ground with a small quantity of washed quartz sand. The fluid is filtered off, the residue is returned to the mortar, and a second extraction is made. This then is also filtered, and finally the combined filtrates are made up to 100 ml. A quantity of this is taken, 1 ml. or more, according to the expected vitamin concentration, and is titrated against the dye. In cases where the extracts are strongly coloured, the method of Lanke (1939) is adopted; an interpolation titration is done in which the dye is extracted with a mixture of xylene and amyl alcohol for the determination of the end point. All estimations were carried out at least in duplicate.

Reagents.—(1) Glass-distilled water, iron- and copper-free.

(2) Metaphosphoric Acid (B.D.H.), a 16 per cent. aqueous solution, kept on ice. If the biological material is very rich in iron, the metaphosphoric acid should be made up in the following buffer:—citric acid 26 0 g., metaphosphoric acid 40 0 g., water 250 ml, under cooling add NaOH 12 0 g. (Lugg, 1942).

(3) 2:6-dichlorophenol-indophenol (B D.H., Roche, Ltd.). Dissolve according to requirement, one to three tablets in 100 ml. of a borate buffer of pH 7 4. The solution on ice will keep its titre for 10 to 14 days, provided care is taken not to contaminate it. Where possible, the solution should be checked against pure ascorbic acid. Other methods for checking the dye are given in a previous communication (Fearon and Kawerau, 1943, 1).

(4) HCl, 36 per cent. If the buffered metaphosphoric acid is used, most extracts of biological materials require the addition of some strong acid in order to bring the pH to 1 5 It has been found sufficient to add 1 5 ml. of 36 per cent. HCl to the final extract.

In accordance with the introduction, the results are grouped under two main headings:—(1) Analysis of plant material freshly collected; and (2) Analysis of plant material collected on the Dublin grocery market; the latter includes analyses of plant material before and after different methods of processing.

ANALYSIS OF PLANT MATERIAL FRESHLY COLLECTED.

It will be seen from a glance at Table 1 that no attempt is made to give an entirely complete survey. Cultivated fruits were only analysed if they are consumed to a large extent by the population in the form of jam, and thereby constitute an important source of the vitamin in the dietary. Wild fruits were only analysed from the point of view that other abundant natural sources of the vitamin may be found similar to that already discovered in the rose hip. Except for the potato freshly picked, cultivated vegetables were not analysed, as it was thought that market samples would be sufficiently fresh and would yield more characteristic results as far as the Irish dietary is concerned.

The results given in Table 1 are all in agreement with those published by Fixsen and Roscoe. There is a considerable variance of opinion, however, on the effect of ripening on the vitamin C concentration in the fruit. For tomatoes, Wokes and Organ (1942) state that the vitamin content does not increase on ripening, and Andross (1941) maintains the same for rose hips, when saying that the under-ripe fruit has the highest vitamin value. In all my analyses, the unripe fruit was picked from the same plant as the ripe fruit, and there seems to be fairly uniform evidence that ripening increases the vitamin content; but it also becomes evident that the term "ripeness" requires definition; the cases of the medlar and the walnut are clear evidence in this respect. Accepting every-day terms, the unripe green walnut shell has more

vitamin C than the ripe fruit that is ready to discard the seed. The concentration is at its height just before the formation of the hard shell (M. Pyke, R. Melville and H. Sarson, 1942). The medlar, too, is not declared ripe until the fruit is soft and ready to discard the seed, and at this point its vitamin

TABLE 1.

Date of Analyses.	*Cultivated Fruit.*	Remarks	Vitamin C mg/100 g. of Fresh Fruit— Ripe.	Unripe.
15/7/42	Strawberries	Outdoor	58·6	—
15/7/42	Raspberries	„	30·9	20·1
15/7/42	Loganberries	„	33·1	21·3
10/9/42	Tomatoes	„	15·6	9·2
21/9/42	Tomatoes	„	28·2	—
21/9/42	Tomatoes	„	35·6	—
21/9/42	Tomatoes	Greenhouse	31·7	—
21/9/42	Tomatoes	„	16·6	—
	Wild Fruit.			
8/9/42	Blackberries		15·1	11·6
8/9/42	Haws		5·0	4·2
8/9/42	Sloes		4·8	1·8
20/11/42	Medlars	Ripe—Soft	0·2	
		Unripe—Hard		13·5
23/10/42	Walnut Green Shell		669·8	—
23/10/42	Walnut Green Shell	Same Fruit	550·0	—
23/10/42	Walnut White Kernel	Same Fruit	15·3	—
23/10/42	Walnut Hard Brown Shell	Same Fruit	0·0	—
28/9/42	Wild Rose Hip	Co. Wicklow	415·8	241·7 (8/9/42)
25/9/42	Wild Rose Hip	Co. Dublin	211·2	—
25/9/42	Sweet Briar Hip	Co. Dublin	371·0	190·1
25/9/42	Burnet Rose Hip	Co. Dublin	267·0	—
17/9/43	Wild Rose Hip	Co. Kerry	365·3	—

content is low. From a biochemical point of view, we must recognise that the changes that occur in the flesh of the fruit just prior to the shedding of the seed are degenerative ones. Disintegration of cellular structure rapidly leads to loss of ascorbic acid, hence the "ripe" medlar hardly contains any of the vitamin. Ripeness might thus be defined as the state of the fruit at which maximum development has occurred prior to any preparation for the discarding of the seed. Where maximum development is not accompanied by obvious colour changes, as in the medlar, this point may be difficult to determine; where the fruit is naturally soft, like the tomato, degenerative changes may be impossible to discern at their early onset. One is inclined to believe, then, that this point of definition has an important bearing on the presentation of results.

The cultivated fruit may be considered under two headings: luxury and uitility fruit. Except for loganberries, only the latter class of fruit has been analysed, especially since most of the luxury fruit such as cherries, plums, peaches, etc., contain little of the vitamin. Strawberries and raspberries, although a luxury in the early season, are as a main crop chiefly used by the jam manufacturers, and hence must be considered in this connection with the Irish dietary. A similar statement can be made for the tomato, which is becoming less and less of a luxury food. Table 1 demonstrates the fact that there is no difference between outdoor and greenhouse-grown tomatoes as far as their vitamin C content is concerned.

Not all types of wild fruits from the hills and plains of the 26 counties have been analysed. Samples were collected from time to time, representing the main crops. Rose hips and blackberries must be considered the nation's most important natural store of vitamin C. The results indicate that it would be worth while, in case of shortage of this vitamin for medical purposes, to prepare vitamin concentrates from these sources (as has already been done in some countries). The walnut, although possessing a high vitamin concentration even at the time the nut can be collected, is too scarce a plant to contribute to the Irish dietary. The results given for the rose hips are significantly lower than those found by British workers. Species identification would have been important in this instance since Pyke and Melville (1942) have shown that the range of vitamin concentration is closely related to the species of rose. Further examination showed that the earlier the species ripened the higher was the vitamin concentration (Harrison, Blackburn and Bolton, 1942). According to Dr. D. A. Webb, the commonest varieties in Ireland are *R. canina* and *R. arvensis*. *R. micrantha* occurs in counties Cork and Kerry, and the Burnet Rose (*R. spinosissima*) grows along the coastal mountains; more rarely one finds *R. stylosa* and *R. agrestis*. Of these varieties *R. canina* shows the highest range of vitamin concentration, up to 1·0 per cent in the tables of Pyke and Melville (1942). These data support the claim made by the Russian workers, Iwanoff and Bukin (1937), that the same species of rose shows a greater vitamin concentration if grown in a more northerly climate than in a southerly climate. Rose hips from Scotland have shown values of over 2·0 per cent., and some Russian varieties have as much as 4·0 per cent. The relatively moderate Irish values might find their explanation in the soft climatic conditions preponderant here; a sufficiently large number of samples have not been analysed, however, to justify an opinion on this point.

Other wild fruit not included in the tables might be worth collecting, if abundant in any particular region. Andross (1941) gives values for elderberries 25–30 mg. per 100 g. fresh fruit; bilberries 8–9 mg. per 100 g. fresh fruit; and crab apple 10–12 mg. per 100 g. of fresh fruit. Jam can be made from the berries of the sea buckthorn (*Hippophae rhamnoides*), which may contain as much as 200 mg. ascorbic acid per 100 g. of fresh fruit (Griebel and Hess, 1940), and is a plant native to the northern sea coast not far from Dublin, and along the banks of the Shannon near Athlone (Dr. D. A. Webb).

Analysis of Plant Material collected from the Dublin Market.

Dietary lack of vitamin C is most likely to occur during the latter half of the winter. Random samples of the more common vegetables were taken from the Dublin market, and analysed. Of those poor in vitamin C, only two samples were taken, whilst of those rich in the vitamin, at least 5–10 samples were analysed.

TABLE 2.—Market Vegetables.

Date of Analysis	Vegetable	Vitamin C mg./100 g. fresh plant. Average	Vitamin C mg./100 g. plant. Range given by Fixsen and Roscoe, 1940
2/10/42 18/1/43 3/12/42	Cabbages, all samples, Duke of York Varieties: Savoy and	50·5	20·0–60·0
18/1/43 20/1/43	Brussels Sprouts	70·5	72·0–146·0
20/1/43	Cauliflower	51·3	19·0–101·0
21/1/43	Turnip (yellow, Swede)	26·0	17·0–43·0
22/1/43	Turnip (white)	23·5	
25/1/43	Carrot	10·2	1·0–31·0
25/1/43	Celery	4·8	1·0–5·7
25/1/43	Onions	10·0	2·0–15·0
27/1/42	Horse Radish	27·0	
18/10/42	Kohlrabi, leaves	112·8	
18/10/42	Kohlrabi, tuber	65·2	16·0–100·0

The results in Table 2 are encouraging in that they all tend to reach the upper limit of the range given by Fixsen and Roscoe. For winter vegetables collected from the market, this is a very satisfactory result. The only vegetable included in the table that was not bought on the market is the kohlrabi, a plant unfortunately not grown on the field scale in Ireland. Warne (1942) also reported high vitamin C values for kohlrabi grown in England, and advocated field scale cultivation. This palatable vegetable certainly should be fostered. One has little hope, however, of changing the dietary of the Irish rural population.

ANALYSIS OF IRISH POTATOES.

Scurvy was well known in Ireland after the potato famines of the last century. Even at the present day, the lack of adequate supplies of cabbage in some of the rural districts of Ireland is astounding. To insure healthy potato crops, and an adequate and cheap supply during the early spring, has always been recognised as a policy of vital national importance.

Vitamin C in the potato might have been first suspected by Szent-Györgyi (1925), who obtained a strongly reducing fraction from potato juice three years before he isolated his now famous "hexuronic acid" from the adrenal cortex. Pietsch (1927) did not suspect the antiscorbutic vitamin, either, when he found that potato juice reduces o-dinitrobenzene, a reaction that is now known to be given by vitamin C (Fearon and Kawerau, 1a, 1943). The work of Pelc and Podzimková (1933) is amongst the first for the correct assay of vitamin C in the potato. Many extensive surveys were to follow (Pfankuch, 1935; Pett, 1936; Scheunert *et al.*, 1937; Smith and Paterson, 1937; Steward and Preston, 1940; Smith and Gillies, 1940; Olliver, 1941). War conditions have aroused fresh interest in the potato, as a nearly complete food (Salaman, 1940; Chick, 1940; Lampitt and Goldenberg, 1940).

Two major difficulties attend the assay of vitamin C in the potato by the dye reduction method. The first is described in the work of Smith and Paterson (1937), who have shown, contrary to the earlier work of Pfankuch (1935), that virus-diseased potatoes may contain up to over 50 per cent. more of the vitamin than healthy specimens of the same variety; they go even as far as to rely on vitamin assay for the diagnosis of virus infection. All potatoes analysed by me, except the random market samples, were obtained with the aid of the Department of Agriculture as Government certified virus-free. The second difficulty also arises from diversity of opinion. Pfankuch (1935) and Guthrie (1937) mention a glutathione concentration in the potato of under 10 mg. per 100 g., whereas Pett (1936) reports values as high as 80 mg. per 100 g., which would be 5–8 times as much as the usual average concentration of ascorbic acid, and thus would lead to erroneous values in the dye titration unless most stringent conditions are observed. I have tested a number of samples for glutathione by the procedure recommended by Lugg (1942), and have come to the conclusion that in the final dilutions of potato extract employed the glutathione constitutes a negligible fraction as far as the reduction of the dye is concerned.

Results for Raw Potatoes:—Table 3 shows a typical series of values obtained for the various parts of the potato plant (variety, Kerr's Pink) immediately on harvesting. The highest concentration of ascorbic acid occurs in the leaf of the plant, a fact also recorded by Smith and Gillies (1940); but unfortunately potato leaves are unsuited for human food. As far as the tubers are concerned, the highest concentration for this variety coincides with the tubers of the greatest fresh weight. The exhaustive research of Smith and Paterson (1937) into the vitamin C distribution of the potato gives consideration to the fresh weight of the tuber, but any corresponding relationship to the vitamin concentration is considered by them to be an inconstant and unimportant feature.

A survey including all potato varieties that are grown in Ireland would have been too extensive a work to be included in this survey; all varieties obtainable on the Dublin market were, however, analysed in December, 1942.

TABLE 3.—Freshly Harvested Potato (Kerr's Pink).

Date of Analysis	Part analysed	Total fresh weight. g.	Vitamin C mg./100 g.	Vitamin C mg/100 g. Smith and Gillies, 31/8/1940
11/9/42	Leaves	—	89 5	51 0
"	Stem	—	0 5	15/6/40 10 0–20·0
"	Tuber	85 0	34 0	31/8/40 31·0
"	Tuber	30·0	27·4	
"	Tuber	11 0	23 8	
"	Roots	—	1 0	

After visiting more than 20 different sources of supply, only four different varieties could be obtained. From these, the largest tubers were selected, each weighing over 130 g., and the results are given in Table 4.

TABLE 4.—Market Potatoes, December, 1942.

Variety	Vitamin C mg/100 g. Average of 5 10 Tubers	Vitamin C mg/100 g. Smith and Gillies, 1940
British Queen	17 32	
Kerr's Pink	21 30	12 90
Arran Pilot	14 1	
Arran Chief	13 5	5 70

The ascorbic acid content in potatoes at least 4 months old appears to be very good when compared with the figures of Smith and Gillies (1940). The higher results may be due entirely to the difference of size. Scheunert *et al.* (1936, 1937) in their potato analysis took into consideration the fresh weight of the tuber; unfortunately, however, they only classified them into "large" and "small" ones. For new potatoes, they found in every case a greater concentration of the vitamin in the larger potatoes; in old potatoes, they found this relationship to hold good only for some varieties. Table 5 shows the results of the analysis of a stone of *Kerr's Pink* after four months' storage,

TABLE 5.—Relation of Size to Vitamin Content.

Variety.	Weight in g.	Vitamin C mg./100 g.	Variety.	Weight in g.	Vitamin C mg./100 g.
British Queen	50–100	18·16	*Kerr's Pink*	50–100	13 1
	100–130	18·00		100–130	17 4
		—		130–150	21 3

and a stone of *British Queen* freshly harvested, the potatoes being assorted according to their weight.

The results are average values for an analysis of at least 5 tubers; in some weight groups, ten tubers were analysed. *Kerr's Pink* appears to show significantly greater values for heavy tubers, whether they are freshly harvested (Table 3) or whether they have been stored for four months; the same observation could not be made on *British Queen*. Drew and Deasy (1941), in their analysis of Irish potatoes for their starch content, showed that, speaking for the same variety of potato, a reduction of size resulted in an increased percentage of starch, it is not likely, therefore, that the increased vitamin concentration is correlated with the starch concentration.

A number of investigators (Smith and Paterson, 1937; Ott, 1937; Scheunert *et al.* 1937, 1940) found that climatic conditions and different methods of manuring had very little influence on the ascorbic acid concentration in the potato tuber. Root vegetables differ in this respect from fruit Charley's observations (1942) on blackcurrants grown in controlled plots over a number of years indicate that climatic conditions may have a profound influence on the ascorbic acid content of the fruit, a similar statement has been recorded for rose hips.

Results for Cooked Potatoes.—The survival of vitamin C after varying methods of cooking has been a matter of extensive investigation. The position may be summarized as follows:—Ascorbic acid is fairly well protected in its natural surrounding, especially if the cellular contents include glutathione (Crook and Hopkins, 1938), as obtains in the potato. The vitamin survives freezing (Scheunert and Reschke, 1940), heating to 100° C. or over, as naturally occurs in frying (Lyons and Fellers, 1939), and even drying in air vats (Javillier, 1939; Somogyi, 1943). The amount of sodium bicarbonate commonly added to boiled vegetables does not appear to harm the vitamin (Olliver, 1940). A complete review of the various cooking methods, as given by Miss Olliver (1941), demonstrates as a general fact for all vegetables that the loss of ascorbic acid is one due to extraction by the cooking water, and not so much to actual chemical change. This significant statement is corroborated by the results obtained in my experiments on cooked potatoes. Potato tubers (British Queen), harvested in August, 1943, were sorted according to their fresh weight, and used for the following cooking experiments within the week of lifting. The method most commonly employed in the rural districts of Ireland for cooking potatoes was adhered to. The potatoes were put into cold water to which salt had been added, and were boiled until they became soft on being pricked by a fork, which usually occurred within 20–30 minutes. Peeling of potatoes was performed with a stainless-steel slotted-blade knife. At least four independent analyses in duplicate were made for each sample.

The principles that can be deduced from the results of Table 6 are generally applicable to most vegetables, except for one important difference: all green leafy vegetables contain an enzyme, ascorbic acid oxidase, which is capable of destroying the vitamin rapidly, so rapidly indeed, that, for example, in

the tomato 92 per cent. of the vitamin may be lost in 7 minutes from the time of slicing (Wokes and Organ, 1942). For this reason, green vegetables should be put directly into boiling water or live steam. The potato alone may be put on in cold water, since it does not contain the enzyme (Johnson and Zilva, 1937; Mommaerts, 1943). The more finely a vegetable is cut, the more of the vitamin is lost by enzyme action and by subsequent extraction into the cooking

TABLE 6 (*a*).—Cooked Potatoes.

Unpeeled and uncut before treatment.	Vitamin C mg./100 g.	Peeled and cut into quarters before treatment.	Vitamin C. mg./100 g.
Before Cooking (entire tuber)	17 78	Before Cooking (entire tuber)	18·55
Immediately after Cooking 20–30 minutes†	13·00	Immediately after Cooking 20–30 minutes†	8 90
After 4 Hours keeping in Hot-box†	10 30	Mashed and kept 4 Hours in Hot Box†	3 30
24 Hours after Cooking, Air-cooled†	10 00	Mashed, kept 24 Hours, Air cooled†	0 85
24 Hours after Cooking, re-heated to 50° C. for 15 min.	5 20	—	—
Original Cooking Water. (10 Tubers) pH 5 0–6 0	1 50–2 10	Original Cooking Water. (3 Tubers)	1 35-2 88

† Edible portion only.

TABLE 6 (*b*).—Cooked Potatoes. Balance Sheet.

Unpeeled and Uncut Potatoes. Vitamin C			Potatoes Peeled and Cut into Quarters. Vitamin C		
% Retained.	% Extracted.	% Destroyed.	% Retained.	% Extracted.	% Destroyed.
95 6	1 8	2 6	53 4	10 6	36 0

water (Wellington and Tressler, 1938). Heating beyond the minimum cooking time, keeping hot, and re-heating are practices to be avoided if waste of the vitamin must be prevented (Scheunert and Reschke, 1938; Olliver, 1940).

Occasionally the question is raised as to "bound ascorbic acid" in the potato. Considering the new chemical reaction for dehydroascorbic acid described by Fearon and Kawerau (1, 1a, 1943), the possibility of a bound ascorbic acid that has even at present failed to be identified, must be borne in mind. We are in agreement with Harris and Olliver (1942) and Rolf (1941) in saying that by the ordinary methods of boiling and weak acid hydrolysis, no "bound ascorbic acid" can be detected. Dehydroascorbic acid has been

found in the potato up to a concentration of 5 0 mg per 100 g. of healthy tuber, and this value does not increase during storage at which time the reduced ascorbic acid decreases up to 50 0 per cent. of its original value (Smith and Gillies, 1940).

At times the question is raised as to how much of the vitamin is discarded with the potato peels. In Table 5 I reported on the vitamin content of Kerr's Pink potatoes, the amount of vitamin for the three weight groups ranged between 13·1 and 21 3 mg. per 100 g. Raw potatoes belonging to the identical weight groups were peeled as described, and the vitamin content of the very thin skins was estimated. For all three weight groups the vitamin content of the skins was practically identical, the range being: 8 0–9 0 mg. per 100 g.

ANALYSIS OF PRESERVED FOODS.

The next most important items as far as the vitamin C content of the Irish dietary is concerned, are jams. At some times of the year, particularly during the emergency, jam is more plentiful than butter, and must be considered an important supplement to the vitamin C intake, especially in young children. Methods of preserving food differ, and no matter how extensive a survey is made, the loss of the vitamin by destruction can never be accurately predicted unless every step is taken to secure optimum conditions for preserving the vitamin. The whole problem of protection of ascorbic acid has been dealt with in a previous paper, and for the present I wish only to emphasise one of the main items, namely, the significance of copper utensils used in preserving fruit. The organic matter of the fruit offers fair protection to the vitamin if traces of copper are involved, but the amount of copper removed by the fruit acid from a copper preserving pan is sufficient to destroy more than 50 per cent. of the ascorbic acid during the short period of heating. The results given in Table 7 make this point obvious; in both experiments the same crop of rose hips was used.

TABLE 7.—Vitamin Destruction by Copper. Wild Rose Hips.

Lot 1 in Copper Pan. *Method.*	Lot 2 in Enamelled Pan. *Method.*
Rose Hips 2 lb.; Sugar 1 lb. 2 oz. Hips washed and put into Copper Pan, covered with water and brought to boil and simmered until tender, about 20 min. Pulp was then pressed and residue again covered with water and brought to boil momentarily in Copper Pan. Pulp again pressed and juice returned to Copper Pan, when sugar was added and syrup boiled for 5 minutes.	Rose Hips 2 lb., Sugar 1 lb 2 oz. Hips washed and crushed and put into Enamelled Pan and covered with one pint of water. T = 70° C. Stood for 12 hours. A further pint of boiling water added and again stood for 12 hours. The mash was then pressed and the juice returned to Enamelled Pan and the sugar was added. The syrup was now boiled for 5 minutes. The final volume was the same as in Lot 1.
Ascorbic Acid: 35·2 mg/100 g.	Ascorbic Acid: 98 0 mg /100 g

It has generally been claimed that where ideal conditions prevail, commercial methods of fruit preserving give better vitamin returns than home methods (Olliver, 1940; Lueck and Pilcher, 1941), especially where canning is resorted to. The final vitamin concentration must depend on the initial vitamin concentration; so if home preserving starts at the same level as commercial preserving, working with the same precautions it can achieve a higher final vitamin concentration, because during home preserving economic considerations need not restrict the final fruit concentration of the jam to approximately 60 per cent. The high ascorbic acid values given in Table 8 as

TABLE 8.—Home-made Fruit and Vegetable Extracts.

Date	Material analysed	Vitamin C mg/100 g.	Method of Preparation.
21/7/42	Syrup of Blackcurrants	49 3	10 oz Water + 5 oz. Fresh Fruit. The Fruit is put straight into the quick boiling water; boiled 5 min., strained, no sugar.
21/7/42	Syrup of Blackcurrants	40 5	10 oz. Water + 5 oz. Fresh Fruit Fruit crushed and left standing for 15 min., then treated as before
17/7/42	Syrup of Blackcurrants	143 0	10 oz. Water + 56 oz. Market Fruit. Crushed and hot water added. Sterilized at 160° F. for 15 20 min
17/7/42	Syrup of Blackcurrants	100 1	10 oz. Water + 56 oz. Market Fruit (different lot from previous sample) Crushed after heating at 160° F. for 15–20 min.
Oct. 1942	Syrup of Elderberries	13 2	1 0 oz. of Water to 3 0 oz. of Fruit. Boiled 3 min Fruit crushed while coming to the boil. No sugar. Analysis 5 days after prep.
Oct. 1942	Juice of Cabbage	20 0.	Boiled to softness with ½ quantity by weight of water. Analysis 3 days after prep.
Oct. 1942	Juice of Swede Turnip	0 4	Boiled to softness with ½ quantity by weight of water. Analysis 3 days after prep.

* Except for the vitamin analyses, these cooking experiments were carried out by Dr. Ella Webb.

compared with the low values given in Table 9 for the vitamin concentration in blackcurrant jam, must be interpreted on these lines.

TABLE 9.—Commercial Fruit and Vegetable Extracts.

Date	Material analysed	Vitamin C mg/100g.	Manufacturers	No. of Jars analysed
Oct. 1941	Orange Marmalade	9·3	A	6
Oct. 1941	Juice of Canned Whole Oranges	49 0	E	4
Oct. 1941	Juice of Canned Whole Carrots	3 7	E	2
Nov. 1941	Rose Hip Syrup	57 0	B	4
Nov. 1941	Rose Hip Syrup	47 2	C	2
Nov. 1941	Rose Hip Syrup	68 0	D	1
Nov. 1941	Rose Hip Syrup	67 3	A	3
Oct. 1942	Rose Hip Syrup	113 0	A	4
Oct. 1941	Blackcurrant Jam	25 2	A	4
Sept. 1942	Blackcurrant Jam	35 1	A	3
Sept. 1942	12 months old Canned Blackcurrant Purée	34 8	(English)	1

It is interesting to note in Table 8 that crushing of the fruit before heating results in loss of the vitamin, and how, by increasing the fruit concentration, values for ascorbic acid can be obtained that are far higher than those reached in commercial production.

Commercial rose hip concentrates in 1941 were very poor in their vitamin content when compared with the values, 100 0–200 0 mg. per 100 g., given in the English literature (Andross, 1941). The local manufacturers allowed me to investigate their process to determine at which stage the loss of vitamin occurred. Over 50 samples were analysed, and in accordance with the findings the process was modified, giving the satisfactory result in 1942 of an at least 50 per cent. increase in the vitamin content of Irish manufactured rose hip syrups.

The suggestion has been made that it might be necessary to dispense rose hip syrup through the hospital dispensaries, in case importation of the synthetic vitamin should fail altogether. The question would then arise as to how fast the vitamin deteriorated in an opened jar of rose hip syrup. It will seen from Table 10 that the loss after the first month is not more than 10 per cent.; practically 50 per cent. is lost after 4 months, and a slight amount of fermentation has set in after this length of open storage, a thing not likely to occur in busy dispensaries.

Unfortunately no data are available for the rate of deterioration of vitamin C in unopened jars of rose hip syrup. Wokes *et al.* (1942) compute the loss of ascorbic acid from rose hips as 1 per cent. per week at 5·0° C., with a coefficient of 2 0 for each 10 0° C. rise. At storage temperature of 15·0° C., the average, the loss therefore is only slightly greater than that in the fresh fruit.

TABLE 10.—Deterioration of Vitamin C on Keeping.

Description	Remark	Vitamin C Content in mg./100 g. Fresh	After One Month	After 2 M.	After 4 M.	After 12 M.	After 18 M.
Rose Hip Syrup (Firm A, 1941) 3 Jars analysed	Clear Glass Jars stored in cupboard (left open)	53·5	48·0	43·0	26·5	—	—
(°/₀ of Vitamin deteriorated)		—	(10·2%)	(19 5%)	(49 9%)	—	—
Rose Hip Jelly Firm A, 1942)	Jars as above (Only unopened Jars analysed)	61·1	—	—	—	12·0	—
(% of Vitamin deteriorated)		—	—	—	—	(80 3%)	—
Orange Marmalade (Firm A, 1941)	Jars as above (Only unopened Jars analysed)	9·3	—	—	8 5	—	5·0
(% of Vitamin deteriorated)		—	—	—	(8 6%)	—	46·2%

Analysis of the Cost of Vitamin C.

The vitamin C requirement of the healthy adult human being has been assessed by independent workers (Review: Brit. Med. J., 1942, p. 227), and is accepted at the level of 50 mg. per day. Based on this fact, a table was compiled by Miss Olliver in 1940 showing the cost of 50 mg. of vitamin C, as derived from various sources of cooked and preserved foods. A table of this nature, of course, is only of topical interest, since the cost of the different foods varies enormously with the seasons and with the general economic conditions of the country, especially during a state of emergency. Table 11 is similar to that of Miss Olliver's; where applicable her prices relating to the English market of the autumn of 1940 are included in the table.

TABLE 11.—Comparative Cost of Ascorbic Acid in Eire.

Material	Cost of 1 lb.	Vitamin C mg./100 gm.	Amount required to give 50 mg.	Vitamin C Cost of 50 mg.
Potatoes Boiled unskinned	1½d.	13·0	13 1/3 oz.	1d.
Potatoes Boiled skinned	1½d.	8·9	1 lb. 3½ oz.	1½d.
M. Olliver (1940) Potatoes Home cooked	1½d.	8·0	1 lb. 6 oz.	1½d.
Cabbage Juice	2d	20·0	8¾ oz.	1d.
M. Olliver (1940) Cabbage Home cooked	2d.	21·0	8½ oz.	1d.
Elderberry Syrup	Sugar 5½d.	13·2	13 oz.	4½d.
Home made Blackcurrant Jam	Fruit 1s. Sugar 5½d.	143·0	1 oz.	1d.
Commercial Blackcurrant Jam	11½d.	37·2	4½ oz.	2¾d.

TABLE 11—*continued.*

Material	Cost of 1 lb.	Vitamin C mg./100 gm.	Amount required to give 50 mg.	Vitamin C Cost of 50 mg.
Home Made Rose Hip Syrup	Sugar 5½d.	98·0	1¾ oz.	¾d.
Commercial Rose Hip Syrup	2s	113 0	1½ oz.	2¼d.
Commercial Marmalade	1s. 7d.	9 3	1 lb. 2¾ oz.	1s. 9½d.
Tomatoes	2s.	28·2	6¼ oz.	9¾d.
M. Olliver (1940) Tomatoes	10d.	24·0	7¼ oz.	4½d.
Synthetic Vitamin C	£27	One Tablet 50 mg.	One Tablet	¾d.

The calculations for the main food items, cabbage and potatoes, agree well with those of Miss Olliver. Clearly one does not often eat the amounts necessary of these foods, to obtain all the daily requirement, but in the rural districts where potatoes are always cooked in their skins and eaten in great quantities, no fear need be entertained as to inadequate supplies of the vitamin. The only source of the vitamin that equals the synthetic product in cheapness is home-made rose hip syrup. The notion that one can obtain all the vitamin necessary by taking a little marmalade daily (a popular idea) is a costly delusion, as the table well illustrates.

SUMMARY.

A limited survey of the ascorbic acid content of Irish grown Fruits (wild and cultivated) and Vegetables is presented.

Special attention has been paid to potatoes, rose hips, and blackcurrants.

The effect of cooking on the vitamin was studied by preparing potatoes in a variety of ways. For the better preservation of ascorbic acid, it was found that potatoes should be steamed or boiled in their skins; that most of the vitamin was lost if the potatoes were skinned previous to boiling, if they were re-heated or kept in a hot-box.

The effect of home and commercial preserving methods on the vitamin have been studied. The apparent advantage of home preserving is that a higher fruit concentration can be obtained. Wild fruits can be collected free, with the result that the jam, and with it the vitamin, costs little. The advantage of commercial preparations is founded in the better keeping properties of the material.

In conclusion I wish to express my gratitude to Professor W. R. Fearon for much helpful criticism and advice, and Dr. D. A. Webb and Dr. Ella Webb for their kind collaboration; also to Mr. T. D. McKeever, B.A., and Mr. J. Harwood of this department, for assisting with some of the estimations.

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THE

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alkaline; and (4) substances that act as anti-catalysts, either by depressing the ionisation of copper present in the solution, or by combining with "ascorbic oxidases."

1. *Vitamin Stabilisers.*—Although Scarborough and Stewart (1937) claim that ascorbic esters occur in urine, little is known definitely about such compounds (Fearon and Kawerau, 1943), apart from metallic derivatives such as those used in the original isolation of the vitamin (Szent Györgyi, 1928) but of no significance in the normal diet. Dehydroascorbic acid, however, has been shown to form compounds with glutathione (Bersin *et al.*, 1935), amino acids (Abderhalden, 1938), and thiols, in general (Drake *et al.*, 1942). Results for thiourea are given in Table 1, which shows the iodine titration values of fresh mixtures containing dehydroascorbic acid in buffered solution.

TABLE 1.—Stabilisation of Dehydroascorbic Acid by Thiourea.

Dehydroascorbic Acid: 50·0 mg. per 100 ml. In Borate Buffer, pH 6·5–7·0.
Thiourea: M/100. *Iodine*: N/100. *Temperature*: 15° C.

Dehydroascorbic Acid, in ml. A	Thiourea in ml. B	Iodine Value for A + B, in ml.	Difference of Iodine Value, in ml., due to A
0·00	1·00	0·95	——
1·00	1·00	0·70	0·25
0·00	2·00	1·68	——
2·00	2·00	1·26	0·42

NOTE: Dehydroascorbic Acid alone does not titrate with Iodine.

These data show that thiourea is capable of rapidly combining with and protecting dehydroascorbic acid in aqueous solution. Similar protection has already been found to be given by other thiol compounds (Drake, Smythe and King, 1942).

2. *Reducing Agents.*—At least three reagents are known to be capable of reducing dehydroascorbic acid, and keeping it reduced in ordinary aqueous solutions, namely: (1) hydrogen sulphide (Szent-Györgyi, 1928); (2) stannous chloride in absence of free oxygen (Meiklejohn and Stewart, 1941); and (3) glutathione in presence of tissue extracts (Mawson, 1935; Hopkins and Morgan, 1936; Crook, 1941). We have not been able to confirm the claim (Jorissen, 1941) that glucose in neutral solution reduces dehydroascorbic acid in absence of free oxygen. Other reducing substances capable of protecting ascorbic acid, such as uric acid and thiol compounds, appear to work in virtue of their anticatalytic rather than their reducing properties.

3. *Acid Buffers.*—Owing to the stability of ascorbic acid in solutions of low pH, inorganic acids have been widely used to extract the vitamin from its natural sources, but, as Mapson (1941) has shown, many samples of these acids, even after repeated purification, still contain sufficient copper to catalyse the extracted vitamin. Metaphosphoric acid, however, in high concentration completely depresses the ionisation of copper (Hinsberg, 1937), and for this reason is now generally employed as an extractant. Its instability is its first disadvantage, as on keeping it changes into the relatively ineffective ortho-phosphoric acid, a transformation that can be retarded for at least 15 days by keeping the solutions at 5° C. (Bessey, 1938). Another disadvantage comes from the fact that ferrous iron increases in reducing potential when in presence of metaphosphoric acid, and will bleach the indicator 2 : 6-dichlorophenol-indophenol used in estimating ascorbic acid. This may be overcome by adding citric acid to the metaphosphoric acid, when extracting materials rich in iron (Lugg, 1942).

4. *Anti-catalysts.*—Reduced ascorbic acid is stable in tissue fluids and in natural and processed food materials containing appreciable quantities of copper. This stability is due to the presence of substances that form copper compounds of a low degree of ionisation. Such substances include: glutathione (Hopkins, 1929); thioglycollic acid—β-amino naphthalide (Berg, 1935); sodium diethyl-dithiocarbamate (Eden and Green, 1940); thiourea (Kohnschütter, 1903; McCarthy *et al.*, 1939); proteins and amino acids (Abderhalden and Schnitzler, 1927, 1927a; Borsook and Thimann, 1932); 8-hydroxyquinoline (Barron *et al.*, 1936; Sendroy and Miller, 1939); purines (Giri and Krishnamurthy, 1941); and halogen salts (Mapson, 1941).

Since copper is an invariable micro-constituent of all plant and animal tissues investigated, one or more natural protectors must be available in the organism to preserve the vitamin from catalytic destruction. If this be so, it should be possible to find a non-toxic substance with a high protective value, and capable of being applied both to the stabilisation of the vitamin within the organism and also in industrial preparations and food products. This was investigated by estimating the effect of various possible inhibitors on the catalytic oxidation of ascorbic acid in presence of known concentrations of copper. The first group of substances tested included thiourea and the chief non-colloidal solutes of normal blood plasma. The reagents employed were all of "analytically pure" quality, and the water was "glass-distilled." To suppress the error arising from varying contamination of the reagents by unavoidable traces of copper, and the necessity for frequent estimations, the amount of copper added in each experiment was at least a thousand times greater than that specified to be present in the regents. The reaction of the mixtures was kept at the specified levels, pH 6·4, and pH 6·8, by borate-phosphate buffers, checked from time to time. The ascorbic acid used was in crystalline form, as supplied by Messrs. Roche, and was estimated both by iodine and by iron titration (Fearon and Kawerau, 1943).

TABLE 2.—Protection of Ascorbic Acid from Oxidation in Presence of Copper.

Ascorbic Acid: 17·5 mg. per 100 ml. *Copper*: 2·5 mg. Cu^{++} per 100 ml. *Substances tested*: M/100. *Iron standard*: 1 ml. (= 0 1 mg. Fe^{+++}) is equivalent to 0·157 mg. ascorbic acid. *Temperature*: 15° C. *Reaction*: pH 6·4.

Substance tested.	Ascorbic Acid in mg. per 100 ml. At start	24 hours	48 hours	48 hours + Cu^{++}
Glass-distilled water ...	17·5	4 4	0 0	0 0
Urea	17 5	13 8	9 4	4 1
Uric acid	17·5	10·0	2 5	1·2
Glucose	17·5	9 7	4·1	3·1
Creatinine	17·5	15 1	3 1	3·1
Glutamic acid	17 5	10·0	4 1	0 9
Thiourea	17·5	17·5	17·3	17·3

The total volume of each mixture at the start of an experiment was 11 ml. At the end of 24 hours' incubation, each mixture was divided into two equal parts, to one of which distilled water containing the Cu^{++} was added, the other being diluted to the same extent with distilled water alone.

Control experiments showed that none of the substances tested had any reducing effect on the iron standard used in the titrations, both at the start and at the end of 48 hours' incubation.

A similar set of experiments was carried out in which the substances tested were present in concentrations of the same order as that found in normal blood plasma. In addition, the protective effects of hesperidin and of thiourea were examined.

TABLE 3.—Protection of Ascorbic Acid from Oxidation in Presence of Copper.

Ascorbic Acid: 35·0 mg. per 100 ml. *Copper*: 1·25 mg. Cu^{++} per 100 ml. *Temperature*: 15° C. *Reaction*: pH 6 8. *Total volume of mixture*: 20 0 ml.

Substance tested.	Concentration in mixture, %	Ascorbic Acid, in mg. per 100 ml. at start	after 24 hours	% recovery
Sodium chloride ...	0 5	35·0	0·0	0 0
Glucose	0·1	35·0	4·7	13 4
Urea	0·03	35·0	3·1	8 9
Glutamic acid ...	0·073	35·0	11·8	33 7
Uric acid	0·005	35·0	7 3	20 8
Creatinine	0 000,5	35·0	19·6	56·0
Distilled water ...		35·0	0·0	0 0
Hesperidin	0·007	35 0	0·0	0·0
Thiourea	0·006	35·0	35 0	100·0

Notes on the Protective Substances.

Sodium Chloride.—In concentrations of the same order as those of blood plasma, sodium chloride has no significant protective effect. To obtain this, it is necessary to have the solution more acid than pH 6 (Mapson, 1941; Mystkowski,

1942), while to attain 100 per cent. protection, sodium chloride concentrations of 12 per cent. are required (Mystkowski and Lasocka, 1939).

Glucose.—At the level of normal blood concentration, glucose is moderately effective, but the property is not a characteristic of the monosaccharide, and similar results have been reported for maltose and sucrose (Wokes *et al.*, 1942). A series of experiments carried out with glucose in presence of insulin, in which the hormone was in the normal blood concentration of 0·01 unit per 100 ml. (Gellhorn *et al.*, 1941), and also in concentration of 1 unit per 100 ml., gave no indication of any enhancement of the glucose protection effect.

Urea.—Protection can be demonstrated at concentrations similar to that in blood, but is too slight to be of obvious significance.

Creatinine.—Giri and Krishnamurthy (1941) report protection up to 100 per cent. in short-time experiments at 30° C. A similar degree of protection is provided by some purines, notably xanthine and uric acid. Creatine is ineffective. Our results are in conformity with these observations, and support the conclusion that creatinine and some of the purines, together with glutathione, are the chief non-colloidal protectors of ascorbic acid in animal tissues.

Hesperidin.—Some of the physiological effects of the as yet unidentified vitamin P may be explained in terms of ascorbic acid protection within the organism. Since hesperidin accompanies, or forms part of, the vitamin P complex, we investigated its protective action, but found it completely ineffective.

Glutamic Acid.—This amino acid was used in concentration equivalent to that of the total amino nitrogen of the blood plasma, and at this level afforded relatively high protection. Mystkowski and Lasocka (1939) report similar results for glycine, leucine, and aspartic acid. The property is probably possessed by all the amino acids and peptides, and is to be referred to their ability to form copper complexes of a low degree of ionisation. The nature of these complexes has been studied by Borsook and Thimann (1932), who find that in approximately neutral solutions, the copper is united to the oxygen of the carboxyl group by primary valencies, and to the nitrogen of the amino group by secondary valencies.

Proteins.—Although the ability of copper to catalyse the oxidation of ascorbic acid is lost when the metal is bound to amino acids or simple peptides, it is retained in some degree when the metal is combined with a protein. The combination usually is feeble, and the copper ions, Cu^{+} and Cu^{++}, can be removed by dialysis (Stotz *et al.*, 1937): conversely, proteins can immobilise the copper ions in a solution, and thus contribute to the protection of ascorbic acid to some extent.

Thiourea.—Of all the simple solutes examined, thiourea was outstanding in its protective power, and gave 100 per cent. in both sets of experiments, a result in confirmation of our earlier work on this compound. Thiourea has not been detected in the animal body. It appears to be of very low toxicity, and may find therapeutic application in circumstances where ascorbic acid stabilisation is required, such as during wound repair (Fearon, 1942).

Stabilisation of Ascorbic Acid in Industrial Food Preparations.

The chief food products rich in ascorbic acid are those made from citrus and other fruits, hence the problem of the stabilisation of the vitamin is of practical value in the domestic and industrial preparation of jam, marmalade, and similar preserves.

The possible significance of copper-contamination during processing has been recognised in an order by the British Food Ministry, according to which manufacturers of fruit concentrates containing vitamin C are required to satisfy the Ministry that their process-plants are constructed so that copper surfaces do not come in contact with the fruit juices (Charley, 1942).

The destructive effect of metallic copper and copper oxide on ascorbic acid is shown in the series of experiments recorded in Table 4. Five gram quantities of copper, in the form of bright turnings 2 mm. broad, copper oxide powder, or copper oxide sticks 5 mm. in diameter, were suspended in 100 ml. of a solution containing 46·0 mg. ascorbic acid, the concentration of which was estimated by titrations over a period of 300 minutes. The reaction of the mixture was pH 6 4, and the temperature was 14 0° C.

Table 4.

	Percentage Ascorbic Acid recovered.		
Time in minutes	Copper foil	Oxide sticks	Oxide powder
0	100	100	100
15	91·1	97·6	66 6
30	85·5	96 6	35·5
60	75·5	88 8	0·0
300	5·5	22·2	0 0

These results suggest that bright copper surfaces are relatively inert at low temperature, and that it may be sufficient for industrial purposes to ensure that the boiling pans are not lined by copper, the effect of copper parts of the "cold" sections of the plant being disregarded. Contamination by ionic copper is likely to be much more troublesome, since the metal is introduced in the water, and carried by the fruit, which is often copper-sprayed. Hence, the problem becomes one of finding an ascorbic acid preservative that will immobilise catalytic metals already present. McFarlane (1936) was the first to suggest that certain reagents used in the estimation of copper and iron might be employed commercially for the protection of vitamin C. He showed that the ascorbic acid content of orange juice suffered no loss by spontaneous oxidation if *aa'*-dipyridyl were added, to combine with the iron, and sodium diethyl dithiocarbamate, to combine with the copper. To be of commercial value, any such protector must conform to four requirements: (1) It must be non-toxic, and harmless to the consumer, over long periods, even in relatively high concentration; (2) it should be tasteless, odourless, and colourless, in the concentrations necessary; (3) complex-formation with the catalytic metals must

take place readily over a wide range of hydrogen ion concentration; (4) the complexes must be stable when exposed to the high temperatures and other conditions of the manufacturing processes.

These requirements greatly restrict the choice of protectors suitable for industrial use, and, in consequence, our attention was directed to only one of them, namely thiourea, as being likely to be of use. Unlike the copper complex formed with diethyl dithiocarbamate the thiourea-copper complex is colourless. In high concentrations, it separates out as a white, silky crystalline precipitate; in the low concentrations used in our experiments, no visible change is seen. For complete fixation of the copper, the thiourea must be present in excess; the ratio being 5 to 1 for copper sulphate (Rathke, 1884), and 3 to 1 for copper chloride (Kohnschütter, 1903). The results indicate that thiourea readily forms a copper complex in cold solutions, both at neutrality and in a high concentration of acid.

The value of thiourea as an anti-oxidant has long been known, and the reagent has been used to keep apples from turning brown on exposure to air (Denny, 1935, 1942). Thiourea is odourless, colourless, and without taste, when in dilute solution. Concentrated solutions have a bitter taste, but there is a considerable degree of difference in the susceptibility of different subjects to the taste of solutions of the same concentration. Feeding experiments carried out at the Boyce Thompson Institute and elsewhere have shown that thiourea is non-toxic to lower animals (Hanzlik and Irvine, 1921; Flinn and Geary, 1942). Medes (1937) reports that thiourea is excreted by the kidney in an unchanged form after administration to human subjects. Ingestion of 0 82 g., led to no detectable disturbance in metabolism, except for a slight rise in the urinary output of cystine. Hartzell (1940) reports that a single dose of 0·0168 g. resulted in no unpleasant symptoms. This evidence for the non-toxicity of thiourea has been confirmed by one of us (E.K.) for doses of one gram. Apparently, thiourea displays none of the pharmacological effects possessed by the substituted thioureas studied by Fastier and Smirk (1943). More work obviously is required on the effect of prolonged administration, and the possible formation of decomposition products during prolonged boiling with fruit extracts, before the general use of thiourea as a protector of vitamin C in large-scale industrial work can be advocated, but of all the compounds investigated it appears to be the most promising.

Natural Thermostable Protectors.—Since some vitamin C survives the various manufacturing processes employed in preserve-making, the presence of natural thermostable protectors is probable. Their existence was first demonstrated by Giri and Krishnamurthy (1940) in vegetable extracts from which ascorbic oxidase had been removed by acetone precipitation. We have observed that boiled vegetable extracts display a similar power of protection, which in some preparations is so complete as to suggest the presence of specific stabilisers, possibly of the thiol class.

Mercaptans have been detected in the distillate obtained by boiling cauliflower and red cabbage (Niemann, 1893), and, according to Rekowski (1893),

may arise by acid hydrolysis of more complex organic sulphur compounds. From the distillate yielded by boiled cabbage, Bach (1942) has obtained a sulphur-containing fraction soluble in light petroleum, and capable of acting as a vitamin C protector. Allyl isothiocyanate and sinigrin were found to act similarly in concentrations as low as 5 7 × 10^{-4} at pH 5·9. No attempt, however, was made to estimate or control the amount of copper present in the mixtures.

This work was extended by studying the relative protective effects of distillates from cabbage and potato, as shown in Table 5. Cabbage distillates contain a volatile thiol compound, the amounts of which were too small for exact identification The compound can be characterised by the fact that it gives a transient violet colour on treatment with nitroprusside and ammonium hydroxide after saturating the solution with ammonium sulphate (Heffter, 1908), while the vapour from the solution at room temperature produces a brown stain on a filter paper moistened with lead acetate. The distillate bleaches standard iodine solutions, and at pH 2 titrates sharply with 2 : 6-dichlorophenol indophenol in a manner similar to ascorbic acid. This observation may explain the alleged four-fold increase in ascorbic acid content that Ahmad (1935) has claimed when cabbage has been boiled for 10 minutes.

Autolysis of cabbage extract at 37° C. for 18 hours does not set free volatile thiols, which is in support of Rekowski's contention that acid hydrolysis is required for their release. Potato juice retains some of its protective properties after boiling, and also yields an active distillate. The factor is present both in the surface layer and skin and also in the central part of the tuber. Unlike cabbage, the distillate gives no thiol reactions, although it slowly bleaches iodine. During the boiling of potatoes the protective factor readily passes into the water, whereas during the boiling of cabbage, it escapes with the steam.

A hundred grams of fresh cabbage leaves were boiled with 250 ml. of tap water. From the time of boiling, the distillate was collected over a period of 30 minutes. The yield, 70 ml., was then diluted to 200 ml., and tested for thiol compounds, as described. For the experiments on ascorbic acid protection, the stock was further diluted 1 : 2 with the buffer solution containing the amounts of ascorbic acid and copper, as shown in the Table.

TABLE 5.—Ascorbic Acid Protection by Vegetable Juices and Distillates.

Ascorbic Acid: 46 mg. per 100 ml. *Temperature*: 14° C. *Reaction*: pH 4·6. Results expressed as percentage of the original ascorbic acid recovered after 18 hours. Corrections have been made for the reducing value of the protective substance added.

Copper,	Cabbage Extract.		Potato Extract.	
mg./100 ml.	boiled juice	distillate	boiled juice	distillate
0·25	——	——	66·3	27·5
0·50	53·3	43·4	44·6	24·4
	64·2	95·2		
5 00	22·3	00·0		

SUMMARY.

1. A classification of the agents capable of protecting ascorbic acid has been made, and their relative efficacy is discussed.

2. The protective effect of the chief non-colloidal solutes of blood plasma has been investigated.

3. The protective effect of thiourea in dilute solution has been found to be greater over a wider range of conditions than any other agent examined. The use of thiourea in the stabilisation of vitamin C is advocated.

4. Problems connected with the industrial preparation of fruit and vegetable products rich in vitamin C are discussed.

5. Volatile thiol compounds have been detected in the distillates obtained when cabbage is boiled, and their value as protectors of ascorbic acid has been confirmed.

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No. 18.

ASCORBIC ACID.

PART 3: THE ASCORBIC ACID CONTENT OF FRUITS AND VEGETABLES GROWN IN EIRE.

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In April, 1940, Fixsen and Roscoe published a series of tables of the vitamin content of human and animal foods. The figures very largely represent values obtained by estimations carried out by the authors themselves, as well as a selection of results chosen from over two hundred references to the world literature. Figures from Ireland are not included in this work of reference, because no data have hitherto been available. The work here presented was designed to fill this gap, and at the same time it was intended to show from what part of the dietary the Irish population received its main supply of vitamin C, in order that during emergency conditions this main supply might be adequately protected.

In connection with the task of estimating ascorbic acid in foods, two complex problems commonly arise. One concerns the methods of detecting and estimating the vitamin; the other the stability of the vitamin in its natural and in artificial environment. Earlier communications to this Society deal with these two problems, respectively (Fearon and Kawerau, 1943, 1944).

It has been already stated that the purpose of this investigation is twofold, and the following will serve to illuminate the issue more clearly. Vitamin C is produced during the growth period of the plant, and one's enquiry may be directed to estimate correctly the number of factors that determine the optimum production of the vitamin in a particular plant. This research, if it covers not one but a number of species, will supply answers to the following questions:—(1) What plants in this country contain the vitamin in the highest concentration? (2) At what time of the year is the highest concentration reached? (3) Does this period coincide with the best time for harvesting a particular plant? (4) Do the customary methods of tilling and soil treatment in this country influence the production of the vitamin favourably or adversely? All these questions relate to one type of enquiry; a second quite distinct line of enquiry would comprise the following questions:—Vitamin C is taken at every meal. Taking all quantitative and qualitative considerations into view, which is the main dietary source of the vitamin of the nation? What is the degree of destruction of the vitamin during transport and distribution to the

town consumer? How is it affected by home and commercial food processing? And, finally, what is the cheapest dietary source of the daily adult human requirements of the vitamin?

The limited facilities at our disposal have made it impossible to answer all the questions just set out. Where personal observations are lacking, and where it is permissible, reference will be made to results obtained by other workers in the same field.

Method of Enquiry.

The principles of representative sampling, as stated by Harris and Olliver (1942), were strictly adhered to where information concerning optimum plant production of the vitamin was desired. These principles include:—(*a*) if different plants are investigated, they should be grown on the same piece of ground; (*b*) if fruit at different stages of ripeness is selected, it should be picked from the same plant, or better still, from the same branch; and (*c*) a minimum of time should elapse between picking the plant, and carrying out the estimation. None of these principles need be observed when the dietary source of the vitamin is the chief concern. Random samples were selected from a number of shops (in Dublin), and the results were pooled. Where different methods of processing of food are studied, it is important to do a sufficiently large number of estimations in order to obtain significant average results characteristic of the particular process adopted.

Method of Analysis.

The considerations given in our first report (Fearon and Kawerau, 1, 1943) and the desire to make the results comparable with those of most other workers led me to choose titration with 2:6-dichlorophenol-indophenol, as the method used in this particular work. The procedure is as follows:—One side of a balance is modified so as to hold in suspension a 2 oz. mortar. The mortar is filled with 20 ml. of strong (16 per cent.) metaphosphoric acid that has been kept on ice. The balance is brought to equilibrium, and from 5 to 20 g. of the substance to be analysed cut with a horn spatula directly into the acid (if cutting is required). The amount taken is weighed, the mortar is removed from the balance, and the substance is thoroughly ground with a small quantity of washed quartz sand. The fluid is filtered off, the residue is returned to the mortar, and a second extraction is made. This then is also filtered, and finally the combined filtrates are made up to 100 ml. A quantity of this is taken, 1 ml. or more, according to the expected vitamin concentration, and is titrated against the dye. In cases where the extracts are strongly coloured, the method of Lanke (1939) is adopted; an interpolation titration is done in which the dye is extracted with a mixture of xylene and amyl alcohol for the determination of the end point. All estimations were carried out at least in duplicate.

Reagents.—(1) Glass-distilled water, iron- and copper-free.

(2) Metaphosphoric Acid (B.D.H.), a 16 per cent. aqueous solution, kept on ice. If the biological material is very rich in iron, the metaphosphoric acid should be made up in the following buffer:—citric acid 26 0 g., metaphosphoric acid 40 0 g., water 250 ml., under cooling add NaOH 12 0 g. (Lugg, 1942).

(3) 2 : 6-dichlorophenol-indophenol (B.D.H., Roche, Ltd.). Dissolve according to requirement, one to three tablets in 100 ml. of a borate buffer of pH 7·4. The solution on ice will keep its titre for 10 to 14 days, provided care is taken not to contaminate it. Where possible, the solution should be checked against pure ascorbic acid. Other methods for checking the dye are given in a previous communication (Fearon and Kawerau, 1943, 1).

(4) HCl, 36 per cent. If the buffered metaphosphoric acid is used, most extracts of biological materials require the addition of some strong acid in order to bring the pH to 1 5. It has been found sufficient to add 1 5 ml. of 36 per cent. HCl to the final extract.

In accordance with the introduction, the results are grouped under two main headings:—(1) Analysis of plant material freshly collected; and (2) Analysis of plant material collected on the Dublin grocery market; the latter includes analyses of plant material before and after different methods of processing.

ANALYSIS OF PLANT MATERIAL FRESHLY COLLECTED.

It will be seen from a glance at Table 1 that no attempt is made to give an entirely complete survey. Cultivated fruits were only analysed if they are consumed to a large extent by the population in the form of jam, and thereby constitute an important source of the vitamin in the dietary. Wild fruits were only analysed from the point of view that other abundant natural sources of the vitamin may be found similar to that already discovered in the rose hip. Except for the potato freshly picked, cultivated vegetables were not analysed, as it was thought that market samples would be sufficiently fresh and would yield more characteristic results as far as the Irish dietary is concerned.

The results given in Table 1 are all in agreement with those published by Fixsen and Roscoe. There is a considerable variance of opinion, however, on the effect of ripening on the vitamin C concentration in the fruit. For tomatoes, Wokes and Organ (1942) state that the vitamin content does not increase on ripening, and Andross (1941) maintains the same for rose hips, when saying that the under-ripe fruit has the highest vitamin value. In all my analyses, the unripe fruit was picked from the same plant as the ripe fruit, and there seems to be fairly uniform evidence that ripening increases the vitamin content; but it also becomes evident that the term "ripeness" requires definition; the cases of the medlar and the walnut are clear evidence in this respect. Accepting every-day terms, the unripe green walnut shell has more

vitamin C than the ripe fruit that is ready to discard the seed. The concentration is at its height just before the formation of the hard shell (M. Pyke, R. Melville and H. Sarson, 1942). The medlar, too, is not declared ripe until the fruit is soft and ready to discard the seed, and at this point its vitamin

TABLE 1.

Date of Analyses.	*Cultivated Fruit.*	Remarks	Vitamin C mg/100 g. of Fresh Fruit – Ripe.	Unripe.
15/7/42	Strawberries	Outdoor	58 6	—
15/7/42	Raspberries	"	30 9	20 1
15/7/42	Loganberries	"	33 1	21·3
10/9/42	Tomatoes	"	15 6	9 2
21/9/42	Tomatoes	"	28 2	—
21/9/42	Tomatoes	"	35 6	—
21/9/42	Tomatoes	Greenhouse	31 7	—
21/9/42	Tomatoes	"	16·6	—
	Wild Fruit.			
8/9/42	Blackberries		15 1	14 6
8/9/42	Haws		5 0	4 2
8/9/42	Sloes		4 8	1 8
20/11/42	Medlars	Ripe—Soft	0 2	
		Unripe—Hard		13 5
23/10/42	Walnut Green Shell		669·8	—
23/10/42	Walnut Green Shell	Same Fruit	550 0	—
23/10/42	Walnut White Kernel	Same Fruit	15 3	—
23/10/42	Walnut Hard Brown Shell	Same Fruit	0·0	—
28/9/42	Wild Rose Hip	Co. Wicklow	415 8	241 7 (8/9/42)
25/9/42	Wild Rose Hip	Co. Dublin	211 2	—
25/9/42	Sweet Briar Hip	Co. Dublin	371 0	190·1
25/9/42	Burnet Rose Hip	Co. Dublin	267 0	—
17/9/43	Wild Rose Hip	Co. Kerry	365·3	—

content is low. From a biochemical point of view, we must recognise that the changes that occur in the flesh of the fruit just prior to the shedding of the seed are degenerative ones. Disintegration of cellular structure rapidly leads to loss of ascorbic acid, hence the "ripe" medlar hardly contains any of the vitamin. Ripeness might thus be defined as the state of the fruit at which maximum development has occurred prior to any preparation for the discarding of the seed. Where maximum development is not accompanied by obvious colour changes, as in the medlar, this point may be difficult to determine; where the fruit is naturally soft, like the tomato, degenerative changes may be impossible to discern at their early onset. One is inclined to believe, then, that this point of definition has an important bearing on the presentation of results.

The cultivated fruit may be considered under two headings: luxury and uitility fruit. Except for loganberries, only the latter class of fruit has been analysed, especially since most of the luxury fruit such as cherries, plums, peaches, etc., contain little of the vitamin. Strawberries and raspberries, although a luxury in the early season, are as a main crop chiefly used by the jam manufacturers, and hence must be considered in this connection with the Irish dietary. A similar statement can be made for the tomato, which is becoming less and less of a luxury food. Table 1 demonstrates the fact that there is no difference between outdoor and greenhouse-grown tomatoes as far as their vitamin C content is concerned.

Not all types of wild fruits from the hills and plains of the 26 counties have been analysed. Samples were collected from time to time, representing the main crops. Rose hips and blackberries must be considered the nation's most important natural store of vitamin C. The results indicate that it would be worth while, in case of shortage of this vitamin for medical purposes, to prepare vitamin concentrates from these sources (as has already been done in some countries). The walnut, although possessing a high vitamin concentration even at the time the nut can be collected, is too scarce a plant to contribute to the Irish dietary. The results given for the rose hips are significantly lower than those found by British workers. Species identification would have been important in this instance since Pyke and Melville (1942) have shown that the range of vitamin concentration is closely related to the species of rose Further examination showed that the earlier the species ripened the higher was the vitamin concentration (Harrison, Blackburn and Bolton, 1942). According to Dr. D. A. Webb, the commonest varieties in Ireland are *R. canina* and *R. arvensis*. *R. micrantha* occurs in counties Cork and Kerry, and the Burnet Rose (*R. spinosissima*) grows along the coastal mountains; more rarely one finds *R. stylosa* and *R. agrestis*. Of these varieties *R. canina* shows the highest range of vitamin concentration, up to 1·0 per cent. in the tables of Pyke and Melville (1942). These data support the claim made by the Russian workers, Iwanoff and Bukin (1937), that the same species of rose shows a greater vitamin concentration if grown in a more northerly climate than in a southerly climate. Rose hips from Scotland have shown values of over 2·0 per cent., and some Russian varieties have as much as 4·0 per cent. The relatively moderate Irish values might find their explanation in the soft climatic conditions preponderant here; a sufficiently large number of samples have not been analysed, however, to justify an opinion on this point.

Other wild fruit not included in the tables might be worth collecting, if abundant in any particular region. Andross (1941) gives values for elderberries 25–30 mg. per 100 g. fresh fruit; bilberries 8–9 mg. per 100 g. fresh fruit; and crab apple 10–12 mg. per 100 g. of fresh fruit. Jam can be made from the berries of the sea buckthorn (*Hippophae rhamnoides*), which may contain as much as 200 mg. ascorbic acid per 100 g. of fresh fruit (Griebel and Hess, 1940), and is a plant native to the northern sea coast not far from Dublin, and along the banks of the Shannon near Athlone (Dr. D. A. Webb).

Analysis of Plant Material collected from the Dublin Market.

Dietary lack of vitamin C is most likely to occur during the latter half of the winter. Random samples of the more common vegetables were taken from the Dublin market, and analysed. Of those poor in vitamin C, only two samples were taken, whilst of those rich in the vitamin, at least 5–10 samples were analysed.

TABLE 2.—Market Vegetables.

Date of Analysis	Vegetable	Vitamin C mg./100 g. fresh plant. Average	Vitamin C mg./100 g. plant Range given by Fixsen and Roscoe, 1940
2/10/42 18/1/43 3/12/42	Cabbages, all samples, Duke of York Varieties: Savoy and	50·5	20·0–60·0
18/1/43 20/1/43	Brussels Sprouts	70·5	72·0–116·0
20/1/43	Cauliflower	54·3	19·0–101·0
21/1/43	Turnip (yellow, Swede)	26·0	17·0–43·0
22/1/43	Turnip (white)	23·5	
25/1/43	Carrot	10·2	1·0–31·0
25/1/43	Celery	4·8	1·0–5·7
25/1/43	Onions	10·0	2·0–15·0
27/1/42	Horse Radish	27·0	
18/10/42	Kohlrabi, leaves	112·8	
18/10/42	Kohlrabi, tuber	65·2	16·0–100·0

The results in Table 2 are encouraging in that they all tend to reach the upper limit of the range given by Fixsen and Roscoe. For winter vegetables collected from the market, this is a very satisfactory result. The only vegetable included in the table that was not bought on the market is the kohlrabi, a plant unfortunately not grown on the field scale in Ireland. Warne (1942) also reported high vitamin C values for kohlrabi grown in England, and advocated field scale cultivation. This palatable vegetable certainly should be fostered. One has little hope, however, of changing the dietary of the Irish rural population.

ANALYSIS OF IRISH POTATOES.

Scurvy was well known in Ireland after the potato famines of the last century. Even at the present day, the lack of adequate supplies of cabbage in some of the rural districts of Ireland is astounding. To insure healthy potato crops, and an adequate and cheap supply during the early spring, has always been recognised as a policy of vital national importance.

Vitamin C in the potato might have been first suspected by Szent-Györgyi (1925), who obtained a strongly reducing fraction from potato juice three years before he isolated his now famous "hexuronic acid" from the adrenal cortex. Pietsch (1927) did not suspect the antiscorbutic vitamin, either, when he found that potato juice reduces o-dinitrobenzene, a reaction that is now known to be given by vitamin C (Fearon and Kawerau, 1a, 1943). The work of Pelc and Podzimková (1933) is amongst the first for the correct assay of vitamin C in the potato. Many extensive surveys were to follow (Pfankuch, 1935; Pett, 1936; Scheunert *et al.*, 1937; Smith and Paterson, 1937; Steward and Preston, 1940; Smith and Gillies, 1940; Olliver, 1941). War conditions have aroused fresh interest in the potato, as a nearly complete food (Salaman, 1940; Chick, 1940; Lampitt and Goldenberg, 1940).

Two major difficulties attend the assay of vitamin C in the potato by the dye reduction method. The first is described in the work of Smith and Paterson (1937), who have shown, contrary to the earlier work of Pfankuch (1935), that virus-diseased potatoes may contain up to over 50 per cent. more of the vitamin than healthy specimens of the same variety; they go even as far as to rely on vitamin assay for the diagnosis of virus infection. All potatoes analysed by me, except the random market samples, were obtained with the aid of the Department of Agriculture as Government certified virus-free. The second difficulty also arises from diversity of opinion. Pfankuch (1935) and Guthrie (1937) mention a glutathione concentration in the potato of under 10 mg. per 100 g., whereas Pett (1936) reports values as high as 80 mg. per 100 g., which would be 5–8 times as much as the usual average concentration of ascorbic acid, and thus would lead to erroneous values in the dye titration unless most stringent conditions are observed. I have tested a number of samples for glutathione by the procedure recommended by Lugg (1942), and have come to the conclusion that in the final dilutions of potato extract employed the glutathione constitutes a negligible fraction as far as the reduction of the dye is concerned.

Results for Raw Potatoes:—Table 3 shows a typical series of values obtained for the various parts of the potato plant (variety, Kerr's Pink) immediately on harvesting. The highest concentration of ascorbic acid occurs in the leaf of the plant, a fact also recorded by Smith and Gillies (1940); but unfortunately potato leaves are unsuited for human food. As far as the tubers are concerned, the highest concentration for this variety coincides with the tubers of the greatest fresh weight. The exhaustive research of Smith and Paterson (1937) into the vitamin C distribution of the potato gives consideration to the fresh weight of the tuber, but any corresponding relationship to the vitamin concentration is considered by them to be an inconstant and unimportant feature.

A survey including all potato varieties that are grown in Ireland would have been too extensive a work to be included in this survey; all varieties obtainable on the Dublin market were, however, analysed in December, 1942.

TABLE 3.—Freshly Harvested Potato (Kerr's Pink).

Date of Analysis	Part analysed	Total fresh weight. g.	Vitamin C mg./100 g.	Vitamin C mg/100 g. Smith and Gillies, 31/8/1940
11/9/42	Leaves	—	89 5	51 0
„	Stem	—	0 5	15/6/40 10·0–20 0
„	Tuber	85 0	34 0	31/8/40 31 0
„	Tuber	30 0	27·4	
„	Tuber	11 0	23 8	
„	Roots	—	1 0	

After visiting more than 20 different sources of supply, only four different varieties could be obtained. From these, the largest tubers were selected, each weighing over 130 g., and the results are given in Table 4.

TABLE 4.—Market Potatoes, December, 1942.

Variety	Vitamin C mg/100 g. Average of 5–10 Tubers	Vitamin C mg/100 g. Smith and Gillies, 1940
British Queen	17 32	
Kerr's Pink	21 30	12 90
Arran Pilot	14 1	
Arran Chief	13 5	5 70

The ascorbic acid content in potatoes at least 4 months old appears to be very good when compared with the figures of Smith and Gillies (1940). The higher results may be due entirely to the difference of size. Scheunert *et al.* (1936, 1937) in their potato analysis took into consideration the fresh weight of the tuber; unfortunately, however, they only classified them into "large" and "small" ones. For new potatoes, they found in every case a greater concentration of the vitamin in the larger potatoes; in old potatoes, they found this relationship to hold good only for some varieties. Table 5 shows the results of the analysis of a stone of *Kerr's Pink* after four months' storage,

TABLE 5.—Relation of Size to Vitamin Content.

Variety.	Weight in g.	Vitamin C mg./100 g.	Variety.	Weight in g.	Vitamin C mg./100 g.
British Queen	50–100	18 16	*Kerr's Pink*	50–100	13·1
	100–130	18 00		100–130	17 4
		—		130–150	21 3

and a stone of *British Queen* freshly harvested, the potatoes being assorted according to their weight.

The results are average values for an analysis of at least 5 tubers; in some weight groups, ten tubers were analysed. *Kerr's Pink* appears to show significantly greater values for heavy tubers, whether they are freshly harvested (Table 3) or whether they have been stored for four months; the same observation could not be made on *British Queen.* Drew and Deasy (1941), in their analysis of Irish potatoes for their starch content, showed that, speaking for the same variety of potato, a reduction of size resulted in an increased percentage of starch; it is not likely, therefore, that the increased vitamin concentration is correlated with the starch concentration.

A number of investigators (Smith and Paterson, 1937; Ott, 1937; Scheunert *et al.* 1937, 1940) found that climatic conditions and different methods of manuring had very little influence on the ascorbic acid concentration in the potato tuber. Root vegetables differ in this respect from fruit. Charley's observations (1942) on blackcurrants grown in controlled plots over a number of years indicate that climatic conditions may have a profound influence on the ascorbic acid content of the fruit; a similar statement has been recorded for rose hips.

Results for Cooked Potatoes.—The survival of vitamin C after varying methods of cooking has been a matter of extensive investigation. The position may be summarized as follows:—Ascorbic acid is fairly well protected in its natural surrounding, especially if the cellular contents include glutathione (Crook and Hopkins, 1938), as obtains in the potato. The vitamin survives freezing (Scheunert and Reschke, 1940), heating to 100° C. or over, as naturally occurs in frying (Lyons and Fellers, 1939), and even drying in air vats (Javillier, 1939; Somogyi, 1943). The amount of sodium bicarbonate commonly added to boiled vegetables does not appear to harm the vitamin (Olliver, 1940). A complete review of the various cooking methods, as given by Miss Olliver (1941), demonstrates as a general fact for all vegetables that the loss of ascorbic acid is one due to extraction by the cooking water, and not so much to actual chemical change. This significant statement is corroborated by the results obtained in my experiments on cooked potatoes. Potato tubers (British Queen), harvested in August, 1943, were sorted according to their fresh weight, and used for the following cooking experiments within the week of lifting. The method most commonly employed in the rural districts of Ireland for cooking potatoes was adhered to. The potatoes were put into cold water to which salt had been added, and were boiled until they became soft on being pricked by a fork, which usually occurred within 20–30 minutes. Peeling of potatoes was performed with a stainless-steel slotted-blade knife. At least four independent analyses in duplicate were made for each sample.

The principles that can be deduced from the results of Table 6 are generally applicable to most vegetables, except for one important difference: all green leafy vegetables contain an enzyme, ascorbic acid oxidase, which is capable of destroying the vitamin rapidly, so rapidly indeed, that, for example, in

the tomato 92 per cent. of the vitamin may be lost in 7 minutes from the time of slicing (Wokes and Organ, 1942). For this reason, green vegetables should be put directly into boiling water or live steam. The potato alone may be put on in cold water, since it does not contain the enzyme (Johnson and Zilva, 1937; Mommaerts, 1943). The more finely a vegetable is cut, the more of the vitamin is lost by enzyme action and by subsequent extraction into the cooking

TABLE 6 (*a*).—Cooked Potatoes.

Unpeeled and uncut before treatment.	Vitamin C mg./100 g.	Peeled and cut into quarters before treatment.	Vitamin C. mg./100 g.
Before Cooking (entire tuber)	17 78	Before Cooking (entire tuber)	18·55
Immediately after Cooking 20–30 minutes†	13 00	Immediately after Cooking 20–30 minutes†	8 90
After 4 Hours keeping in Hot-box†	10 30	Mashed and kept 4 Hours in Hot Box†	3 30
24 Hours after Cooking, Air-cooled†	10 00	Mashed, kept 24 Hours, Air cooled†	0 85
24 Hours after Cooking, re-heated to 50° C. for 15 min.	5 20	—	—
Original Cooking Water. (10 Tubers) pH 5 0–6 0	1 50-2 10	Original Cooking Water. (3 Tubers)	1 35–2 88

† Edible portion only.

TABLE 6 (*b*).—Cooked Potatoes. Balance Sheet.

Unpeeled and Uncut Potatoes. Vitamin C			Potatoes Peeled and Cut into Quarters. Vitamin C		
% Retained.	% Extracted.	% Destroyed.	% Retained.	% Extracted.	% Destroyed.
95 6	1 8	2 6	53 4	10 6	36 0

water (Wellington and Tressler, 1938). Heating beyond the minimum cooking time, keeping hot, and re-heating are practices to be avoided if waste of the vitamin must be prevented (Scheunert and Reschke, 1938; Olliver, 1940).

Occasionally the question is raised as to "bound ascorbic acid" in the potato. Considering the new chemical reaction for dehydroascorbic acid described by Fearon and Kawerau (1, 1a, 1943), the possibility of a bound ascorbic acid that has even at present failed to be identified, must be borne in mind. We are in agreement with Harris and Olliver (1942) and Rolf (1941) in saying that by the ordinary methods of boiling and weak acid hydrolysis, no "bound ascorbic acid" can be detected. Dehydroascorbic acid has been

found in the potato up to a concentration of 5 0 mg. per 100 g. of healthy tuber, and this value does not increase during storage at which time the reduced ascorbic acid decreases up to 50 0 per cent. of its original value (Smith and Gillies, 1940).

At times the question is raised as to how much of the vitamin is discarded with the potato peels. In Table 5 I reported on the vitamin content of Kerr's Pink potatoes, the amount of vitamin for the three weight groups ranged between 13 1 and 21 3 mg. per 100 g. Raw potatoes belonging to the identical weight groups were peeled as described, and the vitamin content of the very thin skins was estimated. For all three weight groups the vitamin content of the skins was practically identical, the range being: 8 0–9 0 mg. per 100 g.

ANALYSIS OF PRESERVED FOODS.

The next most important items as far as the vitamin C content of the Irish dietary is concerned, are jams. At some times of the year, particularly during the emergency, jam is more plentiful than butter, and must be considered an important supplement to the vitamin C intake, especially in young children. Methods of preserving food differ, and no matter how extensive a survey is made, the loss of the vitamin by destruction can never be accurately predicted unless every step is taken to secure optimum conditions for preserving the vitamin. The whole problem of protection of ascorbic acid has been dealt with in a previous paper, and for the present I wish only to emphasise one of the main items, namely, the significance of copper utensils used in preserving fruit. The organic matter of the fruit offers fair protection to the vitamin if traces of copper are involved, but the amount of copper removed by the fruit acid from a copper preserving pan is sufficient to destroy more than 50 per cent. of the ascorbic acid during the short period of heating. The results given in Table 7 make this point obvious, in both experiments the same crop of rose hips was used.

TABLE 7.—Vitamin Destruction by Copper Wild Rose Hips.

Lot 1 in Copper Pan. *Method.*	Lot 2 in Enamelled Pan *Method.*
Rose Hips 2 lb.; Sugar 1 lb. 2 oz. Hips washed and put into Copper Pan, covered with water and brought to boil and simmered until tender, about 20 min. Pulp was then pressed and residue again covered with water and brought to boil momentarily in Copper Pan. Pulp again pressed and juice returned to Copper Pan, when sugar was added and syrup boiled for 5 minutes.	Rose Hips 2 lb.; Sugar 1 lb. 2 oz Hips washed and crushed and put into Enamelled Pan and covered with one pint of water. T = 70° C. Stood for 12 hours. A further pint of boiling water added and again stood for 12 hours. The mash was then pressed and the juice returned to Enamelled Pan and the sugar was added. The syrup was now boiled for 5 minutes. The final volume was the same as in Lot 1.
Ascorbic Acid: 35 2 mg/100 g.	Ascorbic Acid: 98 0 mg./100 g.

It has generally been claimed that where ideal conditions prevail, commercial methods of fruit preserving give better vitamin returns than home methods (Olliver, 1940; Lueck and Pilcher, 1941), especially where canning is resorted to. The final vitamin concentration must depend on the initial vitamin concentration; so if home preserving starts at the same level as commercial preserving, working with the same precautions it can achieve a higher final vitamin concentration, because during home preserving economic considerations need not restrict the final fruit concentration of the jam to approximately 60 per cent. The high ascorbic acid values given in Table 8 as

TABLE 8.—Home-made Fruit and Vegetable Extracts.

Date	Material analysed	Vitamin C mg/100 g.	Method of Preparation.
21/7/42	Syrup of Blackcurrants	49·3	10 oz. Water + 5 oz. Fresh Fruit. The Fruit is put straight into the quick boiling water; boiled 5 min., strained, no sugar.
21/7/42	Syrup of Blackcurrants	40·5	10 oz. Water + 5 oz. Fresh Fruit. Fruit crushed and left standing for 15 min., then treated as before
17/7/42	Syrup of Blackcurrants	143·0	10 oz. Water + 56 oz. Market Fruit. Crushed and hot water added. Sterilized at 160° F. for 15–20 min.
17/7/42	Syrup of Blackcurrants	100·1	10 oz. Water + 56 oz. Market Fruit (different lot from previous sample). Crushed after heating at 160° F. for 15–20 min.
Oct. 1942	Syrup of Elderberries	13·2	1·0 oz. of Water to 3·0 oz. of Fruit. Boiled 3 min. Fruit crushed while coming to the boil. No sugar. Analysis 5 days after prep.
Oct. 1942	Juice of Cabbage	20·0	Boiled to softness with ½ quantity by weight of water. Analysis 3 days after prep.
Oct. 1942	Juice of Swede Turnip	0·4	Boiled to softness with ½ quantity by weight of water. Analysis 3 days after prep.

* Except for the vitamin analyses, these cooking experiments were carried out by Dr. Ella Webb.

compared with the low values given in Table 9 for the vitamin concentration in blackcurrant jam, must be interpreted on these lines.

TABLE 9.—Commercial Fruit and Vegetable Extracts.

Date	Material analysed	Vitamin C mg/100g.	Manufacturers	No. of Jars analysed
Oct. 1941	Orange Marmalade	9 3	A	6
Oct. 1941	Juice of Canned Whole Oranges	49 0	E	4
Oct. 1941	Juice of Canned Whole Carrots	3 7	E	2
Nov. 1941	Rose Hip Syrup	57 0	B	4
Nov. 1941	Rose Hip Syrup	47 2	C	2
Nov. 1941	Rose Hip Syrup	68 0	D	1
Nov. 1941	Rose Hip Syrup	67 3	A	3
Oct. 1942	Rose Hip Syrup	113 0	A	4
Oct. 1941	Blackcurrant Jam	25 2	A	4
Sept. 1942	Blackcurrant Jam	35 1	A	3
Sept. 1942	12 months old Canned Blackcurrant Purée	34 8	(English)	1

It is interesting to note in Table 8 that crushing of the fruit before heating results in loss of the vitamin, and how, by increasing the fruit concentration, values for ascorbic acid can be obtained that are far higher than those reached in commercial production.

Commercial rose hip concentrates in 1941 were very poor in their vitamin content when compared with the values, 100 0–200 0 mg. per 100 g., given in the English literature (Andross, 1941). The local manufacturers allowed me to investigate their process to determine at which stage the loss of vitamin occurred. Over 50 samples were analysed, and in accordance with the findings the process was modified, giving the satisfactory result in 1942 of an at least 50 per cent. increase in the vitamin content of Irish manufactured rose hip syrups.

The suggestion has been made that it might be necessary to dispense rose hip syrup through the hospital dispensaries, in case importation of the synthetic vitamin should fail altogether. The question would then arise as to how fast the vitamin deteriorated in an opened jar of rose hip syrup. It will seen from Table 10 that the loss after the first month is not more than 10 per cent.; practically 50 per cent. is lost after 4 months, and a slight amount of fermentation has set in after this length of open storage, a thing not likely to occur in busy dispensaries.

Unfortunately no data are available for the rate of deterioration of vitamin C in unopened jars of rose hip syrup. Wokes *et al.* (1942) compute the loss of ascorbic acid from rose hips as 1 per cent. per week at 5 0° C., with a coefficient of 2·0 for each 10·0° C. rise. At storage temperature of 15·0° C., the average, the loss therefore is only slightly greater than that in the fresh fruit.

TABLE 10.—Deterioration of Vitamin C on Keeping.

Description	Remark	Vitamin C Content in mg./100 g. Fresh	After One Month	After 2 M.	After 4 M.	After 12 M.	After 18 M.
Rose Hip Syrup (Firm A, 1941) 3 Jars analysed	Clear Glass Jars stored in cupboard (left open)	53 5	48·0	43·0	26·5	—	—
(°/₀ of Vitamin deteriorated)		—	(10 2°/₀)	(19·5°/₀)	(49 9°/₀)	—	—
Rose Hip Jelly Firm A, 1942)	Jars as above (Only unopened Jars analysed)	61·1	—	—	—	12·0	—
(°/₀ of Vitamin deteriorated)		—	—	—	—	(80·3°/₀)	—
Orange Marmalade (Firm A, 1941)	Jars as above (Only unopened Jars analysed)	9 3	—	—	8 5	—	5·0
(°/₀ of Vitamin deteriorated)		—	—	—	(8 6°/₀)	—	46·2°/₀

Analysis of the Cost of Vitamin C.

The vitamin C requirement of the healthy adult human being has been assessed by independent workers (Review: Brit. Med. J., 1942, p. 227), and is accepted at the level of 50 mg. per day. Based on this fact, a table was compiled by Miss Olliver in 1940 showing the cost of 50 mg. of vitamin C, as derived from various sources of cooked and preserved foods. A table of this nature, of course, is only of topical interest, since the cost of the different foods varies enormously with the seasons and with the general economic conditions of the country, especially during a state of emergency. Table 11 is similar to that of Miss Olliver's; where applicable her prices relating to the English market of the autumn of 1940 are included in the table.

TABLE 11.—Comparative Cost of Ascorbic Acid in Eire.

Material	Cost of 1 lb.	Vitamin C mg /100 gm.	Amount required to give 50 mg.	Vitamin C Cost of 50 mg.
Potatoes Boiled unskinned	1½d.	13·0	13 1/3 oz.	1d.
Potatoes Boiled skinned	1½d.	8·9	1 lb. 3½ oz.	1½d.
M. Olliver (1940) Potatoes Home cooked	1½d.	8·0	1 lb. 6 oz.	1½d.
Cabbage Juice	2d.	20·0	8¾ oz.	1d.
M. Olliver (1940) Cabbage Home cooked	2d.	21·0	8½ oz.	1d.
Elderberry Syrup	Sugar 5½d.	13·2	13 oz.	4½d.
Home made Blackcurrant Jam	Fruit 1s. Sugar 5½d.	143·0	1 oz.	1d.
Commercial Blackcurrant Jam	11½d.	37·2	4½ oz.	2½d.

TABLE 11—*continued.*

Material	Cost of 1 lb.	Vitamin C mg./100 gm.	Amount required to give 50 mg.	Vitamin C Cost of 50 mg.
Home Made Rose Hip Syrup	Sugar 5½d.	98·0	1¾ oz.	¾d.
Commercial Rose Hip Syrup	2s.	113·0	1½ oz.	2¼d.
Commercial Marmalade	1s. 7d.	9·3	1 lb. 2¾ oz.	1s. 9½d.
Tomatoes	2s.	28·2	6¼ oz.	9⅜d.
M. Olliver (1940) Tomatoes	10d.	24·0	7¼ oz.	4½d.
Synthetic Vitamin C	£27	One Tablet 50 mg.	One Tablet	¾d.

The calculations for the main food items, cabbage and potatoes, agree well with those of Miss Olliver. Clearly one does not often eat the amounts necessary of these foods, to obtain all the daily requirement, but in the rural districts where potatoes are always cooked in their skins and eaten in great quantities, no fear need be entertained as to inadequate supplies of the vitamin. The only source of the vitamin that equals the synthetic product in cheapness is home-made rose hip syrup. The notion that one can obtain all the vitamin necessary by taking a little marmalade daily (a popular idea) is a costly delusion, as the table well illustrates.

SUMMARY.

A limited survey of the ascorbic acid content of Irish grown Fruits (wild and cultivated) and Vegetables is presented.

Special attention has been paid to potatoes, rose hips, and blackcurrants.

The effect of cooking on the vitamin was studied by preparing potatoes in a variety of ways. For the better preservation of ascorbic acid, it was found that potatoes should be steamed or boiled in their skins, that most of the vitamin was lost if the potatoes were skinned previous to boiling, if they were re-heated or kept in a hot-box.

The effect of home and commercial preserving methods on the vitamin have been studied. The apparent advantage of home preserving is that a higher fruit concentration can be obtained. Wild fruits can be collected free, with the result that the jam, and with it the vitamin, costs little. The advantage of commercial preparations is founded in the better keeping properties of the material.

In conclusion I wish to express my gratitude to Professor W. R. Fearon for much helpful criticism and advice, and Dr. D. A. Webb and Dr. Ella Webb for their kind collaboration; also to Mr. T. D. McKeever, B.A., and Mr. J. Harwood of this department, for assisting with some of the estimations.

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No. 19.

THE CHEMICAL CONSTITUENTS OF LICHENS FOUND IN IRELAND.

CLADONIA SYLVATICA (L.) HARM. EMEND. SANDST.

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THE lichen *Cladonia sylvatica* (L.) Harm. emend. Sandst. was found growing abundantly on peaty soil on the summit of Howth Hill, Co. Dublin, and was identified for us by Mr. Mackenzie-Lamb, of the Natural History Section of the British Museum, to whom we wish to express our thanks. It bears a very strong resemblance to the lichen *Cladonia impexa* Harm., from which it is distinguished by the morphological feature of the tips being bent in one direction. While Asahina records (Acta Phytochimica, 1934, 8, 52) that *Cladonia impexa* shows a negative reaction to benzidine and p-phenylene diamine, *Cladonia sylvatica* gives a red reaction with an alcoholic solution of p-phenylene diamine, indicating the probable presence of a depsidone of the salazic acid type.

The constituents of the lichen *Cladonia impexa* Harm. have already been described by us (Sci. Proc. R.D.S., 1942, **23**, 6). The predominant constituent is l-usnic acid; the lichen also contains the depside perlatolic acid and the sugar alcohol d-arabitol. There is also present a small amount of a sterol-like substance to which we attributed the alternative formulae $C_{32}H_{50}O_4$ or $C_{32}H_{52}O_4$. In view of the results now recorded in the case of the variety of *Cladonia sylvatica* we reinvestigated this sterol-like substance, and found it to consist principally of the triterpene acid, *ursolic acid*, $C_{30}H_{48}O_3$.

We find that in *Cladonia sylvatica* (L.) Harm. the principal constituents are usnic acid and ursolic acid; the latter was identified by comparison of it and its derivatives with ursolic acid and its derivatives, prepared from the leaves of the Bearberry plant (*Arctostaphylos uva ursi*) which grows extensively on the coast of Clare in the neighbourhood of Ballyvaughan. In contrast to the usnic acid from *Cladonia impexa*, which is pure l-usnic acid, the usnic acid in *Cladonia sylvatica* is only weakly lævo-rotatory, and consists principally of the inactive compound. Ursolic acid, which is a very minor constituent of *Cladonia impexa*, is present in *Cladonia sylvatica* in considerable amounts. On the other hand, the amount of perlatolic acid present in *Cladonia sylvatica* is very much less than in the case of *Cladonia impexa*. We did not succeed in isolating identifiable amounts of sugar alcohol from *Cladonia sylvatica*, but obtained in the acetone extract a small amount of what appeared to be fumaro-protocetraric acid.

EXPERIMENTAL.

Ether extract of lichen.

700 g. of air-dried lichen were rubbed through a ⅜″ sieve and extracted twice with 2½ litres of ether. The extract yielded 10 g. of a green gummy

mass, which was treated with 80 c.c. ether, giving an ether solution A_1 and a residue A_2.

The solution A_1 was shaken with 30 c.c. of 5 per cent. sodium bicarbonate solution; the alkaline solution was acidified, extracted with ether, and the product recovered from the ether crystallised several times from 60/80 petrol, when a very small amount of cream-coloured rosettes were obtained—m.p. 102° C. They gave a reddish-brown colour with ferric chloride, and a negative reaction with bleaching powder. A mixed melt with perlatolic acid showed no depression. The amount obtained was only a small fraction of that obtained from an equal weight of *Cladonia impexa.*

Analysis: 4 280 mg. gave 10·510 mg. CO_2; 2·700 mg. H_2O.
5 20 mg. gave 2·72 mg. AgI.
Found C = 67 0; H = 7 2; OCH_3 = 7·15.
$C_{21}H_{29}O_6 . OCH_3$ requires C = 67 5; H = 7 2; OCH_3 = 6 98.

The residue in the ether solution A_1 insoluble in sodium bicarbonate was an intractable gum which was not further investigated.

The residue A_2 was treated with 100 c.c. chloroform, giving a greenish yellow solution A_3 and a grey residue A_4. The solution was concentrated to 25 c.c. and 50 c.c. alcohol added. On standing yellow needles were deposited, which on recrystallisation from a mixture of choloroform and alcohol melted at 193° C. This product had a specific rotation in chloroform solution of - 20·8, and was inactive usnic acid with a small admixture of the lævo variety.

Analysis: 3 973 mg. gave 9·08 mg. CO_2 ; 1 7 mg. H_2O.
Found C = 62 4; H = 4·75.
$C_{18}H_{16}O_7$ requires C = 62·8; H = 4·6.

The chloroform-insoluble fraction A_4 was washed with a small amount of chloroform to remove traces of usnic acid; it was then taken up in ether, and extracted with 5 per cent. sodium bicarbonate solution, which removed a small amount of material which, on crystallisation from 80 per cent. acetone, gave brown plates m.p. 220° C. with gas evolution. A similar product was found in *Cladonia impexa*; the amount was insufficient for further investigation. The ether solution of the partially purified A_4 gave a grey powder m.p. 240–250° C. which showed no reactions with bleaching powder or ferric chloride. It gave a positive Liebermann test, passing rapidly from purple to blue and finally green. It was dissolved in the minimum quantity of boiling alcohol, and on standing formed a gel which slowly transformed into white lustrous crystals, which were afterwards identified as ursolic acid. On shaking an ethereal solution with 10 per cent. caustic soda a white layer of sodium ursolate formed at the interface.

In order to obtain adequate amounts of ursolic acid 6¼ lbs. of air-dried lichen were extracted with ether; 60 g. of a yellowish green material were thus obtained. This was treated in lots of 25 g. as follows:—25 g. were warmed with 50 c.c. alcohol with vigorous stirring, and allowed to stand overnight. The whole was then filtered, washed with 50 c.c. alcohol, and finally

with 10 c.c. alcohol. The alcohol mother liquors contain most of the gum and perlatolic acid, while the alcohol-insoluble residue consists principally of usnic and ursolic acids. The dried residue (18 g.) was treated with successive quantities of 40 and 25 c.c. hot ligroin to remove some further gummy material and some usnic acid; the remainder of the usnic acid was then removed by boiling with 75 and 50 c.c. chloroform. In this way from the total 60 g. of original product, 36·5 g. of a grey powder were obtained. This was crystallised successively from alcohol and from benzene, when a product m.p. 272° C. was obtained, the mother liquors containing material melting below 250° C. The product was then further purified by sublimation in a high vacuum at 275° C.; the ursolic acid sublimed as a snow-white mass of crystals, a small amount of gummy material forming a more volatile fraction. The crystalline material was further purified by crystallisation from alcohol, and then melted at 280–282° C. This material was used for analysis and in the preparation of derivatives.

Analysis: (1) Air-dried material—
3·772 mg. gave 10·47 mg. CO_2; 3 49 mg. H_2O.
0 662 mg. – 5 846 mg. camphor; 8·8° △.
Found C = 75·9; H = 10 28; M.W. = 488.
$C_{30}H_{48}O_3 . H_2O$ requires C = 75 9; H = 10·55; M.W. = 474.
(2) Dried in vacuum at 110° C.—
3 671 mg. gave 10 62 mg. CO_2; 3 48 mg. H_2O.
Found C = 78·89; H = 10·53.
$C_{30}H_{48}O_3$ requires C = 78·94; H = 10·52.

Ursolic acid phenyl urethane.

0 3 g. ursolic acid was dissolved in 60 c.c. toluene, and to the solution 0 04 c.c. phenyl isocyanate in 2·5 c.c. toluene added; the whole was heated for 10 minutes at 100° C., and then concentrated to 10 c.c.; 25 c.c. warm ligroin were then added, and the white precipitate formed crystallised by dissolving in a small volume of chloroform and adding 6 volumes of ligroin. Large transparent plates of the urethane were obtained, m.p. 235° C. Before analysis the material was dried in vacuum at 110° C.

Analysis: 3·863 mg. gave 10·96 mg. CO_2; 3 26 mg. H_2O.
3·379 mg. gave 0 09 c.c. N at 753 mm. and 24° C.
Found C = 77·3; H = 9·3; N = 3·03.
$C_{37}H_{53}O_4N$ requires C = 77 24; H = 9 2; N = 2·43.

Methyl ursolate.

Methyl ursolate was prepared by interaction of sodium ursolate and methyl iodide according to the method of van der Haar (Rec. Trav. Pays Bas, 1924, **43**, 375, 4). It was also prepared from ursolic acid using diazomethane, silver oxide and methyl iodide, or sodium methoxide and methyl iodide. In all cases a gummy material was obtained, which on crystallisation from 60/80 petrol melted finally at 166° C. and showed no depression on mixing with methyl

ursolate prepared from ursolic acid isolated from the leaves of the Bearberry according to the method of Bilham, Kon, and Ross (J.C.S., 1942, **41**).

Analysis: 3 968 mg. gave 11·44 mg. CO_2; 3·88 mg. H_2O.
4·595 mg. gave 2 89 mg. AgI.
Found C = 78 6; H = 10·7; OCH_3 = 6·76.
$C_{30}H_{47}O_2 . OCH_3$ requires C = 79·15; H = 10·6; OCH_3 = 6·6.

Methyl acetyl ursolate.

0 2 g. of the methyl ester was heated for 1 hour with 0·2 g. sodium acetate and 3 c.c. acetic anhydride. On cooling, the mixture was treated with water, and the acetate recrystallised from alcohol. It melted at 235° C., and showed no depression on admixture with genuine methyl acetyl ursolate.

Diacetyl ursolic acid.

0·3 g. ursolic acid was heated for 1 hour with 3 c.c. acetic anhydride. On standing, a crystalline precipitate was formed, which was collected and dried for three days in vacuum over potash. The material melted at 198° C. and then resolidified, and did not melt again until 285° C. It gives a positive Liebermann reaction.

Analysis: 3·88 mg. gave 10·67 mg. CO_2; 3·29 mg. H_2O.
6·856 mg. required 2·3 c.c. N/100 NaOH.
Found C = 75·1; H = 9 4; Acetyl = 14·4.
$C_{30}H_{46}O_3 . (CH_3CO)_2$ requires C = 75·5; H = 9·6; Acetyl = 15·9.

Monoacetyl ursolic acid.

0 2 g. of the diacetyl compound was heated for 1 hour with 5 c.c. of 70 per cent. alcohol. On filtering and evaporating the alcohol to half bulk white crystals were obtained, which on crystallising from alcohol had m.p. 281° C. not depressed on admixture with monacetyl ursolic acid.

Analysis: 4·081 mg. gave 11·49 mg. CO_2; 3·67 mg. H_2O.
Found C = 77·18; H = 9·9.
$C_{30}H_{47}O_3 . (CH_3CO)$ requires C = 77·1; H = 10·0.

Acetone extract of lichen.

700 g. of lichen, after extraction with ether, were extracted with 2½ litres acetone, and gave 4·7 g. of a green paste; this was warmed with water and filtered. On treating the aqueous extract in the same way as in the case of our investigation of *Cladonia impexa* (*loc. cit.*) there was only a small residue, from which it was not possible to isolate any sugar alcohol. The water-insoluble fraction was warmed with 80 per cent. acetone, and filtered. The solution on standing deposited a small amount of crystals, which were soluble in sodium bicarbonate, gave a purple colour with ferric chloride, and a negative reaction with bleaching powder. They turned brown at 220/230° C., and charred at 260° C. and gave a sublimate of fumaric acid. They were probably fumaro-protocetraric acid.

No. 20.

SEA TROUT OF THE WATERVILLE (CURRANE) RIVER.

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Previous work on the sea trout of the Waterville River (Went and Barker, 1943) had raised two interesting problems connected with these fish. As explained by the authors the samples of sea trout examined were representative only of the spring sea trout runs, the close season for the capture of salmon and trout by means of nets and weirs in that river commencing on 16th July, before the smaller sea trout begin to run in force. The two problems arose from the fact that the calculated smolt length of these spring-running sea trout (mean length 10·4 inches) was exceedingly high, being some 3 or 4 inches longer than smolts from other Irish rivers investigated to date (Nall, 1929, 1930, and 1931). Had the smolts of the later-running sea trout similar high mean lengths was the first question to be solved, and, if so, were sea trout fry or smolts being taken by anglers before their initial descent to the sea in the belief that they were small adult sea trout?

In order to solve this problem arrangements were made for the collection of sets of scales and data from the rod catches of sea trout during the 1942 season.[1] Mr. P. J. Hamilton, Clerk and Inspector to the Waterville Board of Fishery Conservators, kindly undertook to carry out the work of collecting the necessary material. Most of the new material, comprising 845 sets of scales and data, was obtained from the rod catches when they were being displayed at the end of each day in the various hotels in Waterville, Co. Kerry. In this way about one-sixth of the total number of fish caught in 1942 by visiting anglers to Waterville was sampled. As far as possible the various sizes were selected for sampling roughly in the proportion in which they occurred in the catches. The majority of the fish captured by anglers in 1942 were taken from Lough Currane, the other lakes in the Waterville system only being fished to a limited extent owing to difficulties in transport. In Table 1 the distribution of the samples, both in time and locality, has been indicated.

[1] The investigation described by Went and Barker, 1943, was completed towards the end of 1941, but circumstances prevented the completion of the typescript and figures until late in 1942. Arrangements had been made, however, for the collection of the new material before the paper reached its final form.

Length of the smolts.

In order to simplify this report it is proposed to discuss matters somewhat out of their usual order, and to deal firstly with the calculated smolt lengths. As will be explained more fully in a later section the length at the end of every year of life of each maiden or unspawned fish with suitable scales was calculated together with the length of the smolt (see page 207). The mean length of all smolts was 9·85 inches, which is somewhat less than that calculated previously (10·4 inches) for the spring sea trout runs (Went and Barker, 1943, page 95). Nevertheless the order of the mean length of the smolts is the same as that obtained previously. Whilst it is true that the small sea trout (fish in their first post-migration summer) have a lower average smolt length than the earlier-running sea trout, the differences are relatively small. In the previous investigation, as in Nall's (1931), the scales of previously spawned fish were included for measurements, which in itself leads to somewhat inaccurate results. Naturally, when dealing with scales taken from rod-caught fish one is unable to tell the date of entry into a river of the individual fish in question. In order to ascertain whether there was one or more distinct groups or populations (the term "race" is hardly correct in this case) the frequency distribution curve of the smolt lengths for all maiden fish examined, including the small number investigated previously, was plotted (see Fig. 1). It is obvious from this curve that there was no clear differentiation, based on the length of the smolt, into two or more groups, in the sea trout of this river. This being so, the material from the spring-running sea trout described previously and the present material have been pooled, and the following portion of this paper relates to such pooled material.

In addition to the distribution curve for all smolts the individual curves for the length of smolts of fish captured in their first post-migration summer and fish captured at a later period of life (mainly those in their second post-migration summer) were added to Fig. 1. It will be seen that the maiden fish in their second post-migration summer have a somewhat greater mean smolt length than those in their first post-migration summer, the mean lengths being 10·2 inches and 9·7 inches, respectively. At this stage, however, it is advisable to mention that even the grouping of material does not give a true picture of the proportion of each class of fish, because of the variation in the percentage of fish sampled. For example, the catch of sea trout in the nets and weirs seldom reaches 1,000 in any one year, and the number of fish sampled was 433, say very approximately 50 per cent. of the total yearly catch. On the other hand one-sixth or less of the rod catch was sampled. By suitable arithmetical manipulation, however, the data available can be used to obtain a fairly accurate idea of the stocks of sea trout in this river, and in the majority of tables the weighted results have been given. (This has been accomplished by considering that the rod-caught fish were three times more numerous than they actually were in the samples.)

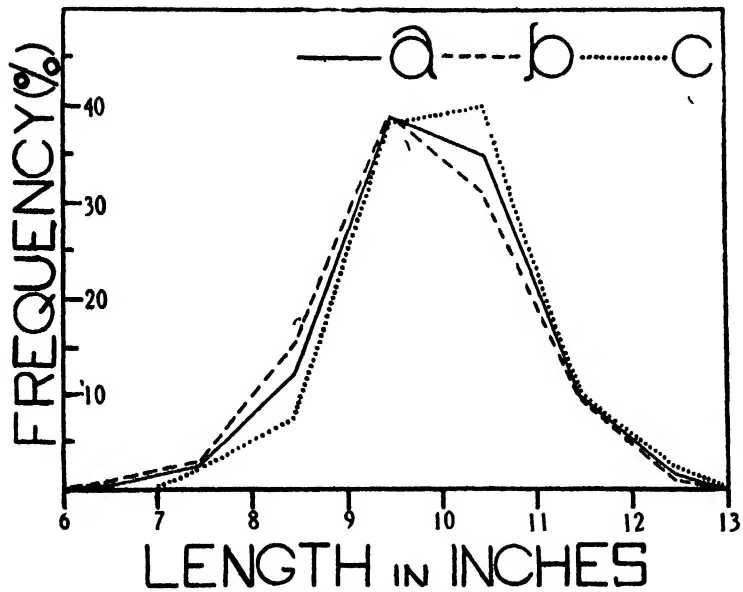

FIG. 1.—Smolt distribution curve.

(*a*) All smolts.
(*b*) Smolts of whitling (maiden fish in their first post-migration summer).
(*c*) Smolts of maiden fish in their second and third post-migration summers.

Are sea trout fry[2] *or smolts being taken by anglers?*

The question as to whether sea trout fry or smolts are being taken unwittingly by anglers in the belief that they are small adult sea trout is the second problem to be solved. Out of 845 sets of scales and data collected in 1942 there were, in addition to a few sets of scales unsuitable for microscopical examination, 39 sets taken from fish which showed on their scales no signs of sea feeding. A number of these fish were obviously brown trout, but there were other sets of scales which may have belonged to either the fry of sea trout or of brown trout. It is naturally impossible to tell from the scales to which category these fish belong, but in 25 cases there was the distinct possibility that the scales had been taken from sea trout fry.

The killing of these fry is, of course, contrary to the provisions of the fishery code, the capture of the fry or smolts of salmon and trout being prohibited by Section 73 of the Fisheries Act of 1842 (5 and 6 Vict. Cap. 106). It is difficult or impossible for an angler to ascertain whether the fish is actually a sea trout smolt without removing the scales. With the possibility of a smolt reaching a length of about 13 inches (the greatest calculated length in these investigations being 12·9 inches) it is only to be expected that the offence of taking sea trout fry might be occasionally committed. This difficulty is minimised to some extent by the enforcement of a by-law on the Waterville River, its

[2] The term "fry" is used here in the legal sense.

lakes and tributaries, prohibiting the killing of any sea trout or "pink" (the local name of sea trout fry) of a size less than nine inches from the tip of the snout to the fork of the tail (By-Law No. 276 dated 16th September, 1909. See Abstract of Fishery By-Laws, 1937). Apart from the fact that a technical offence is being committed by the taking of these large sea trout fry little real harm is being done to the fisheries when one considers the weight of fish involved. If one assumes that the smolts taken average 10 inches in length, and that on their first return to the river from the sea the same fish measure 15 inches, the relative weight of the adult fish to that of the smolt would be very roughly $1{\cdot}5^3$ to 1 or 3·4 to 1. This, however, ignores the possible mortality between the smolt and adult stage, so that taking into consideration the fact that relatively small numbers of these fish are probably taken each year little damage to the fisheries is likely to result therefrom. One cannot, however, apply this argument to the taking of sea trout fry in other rivers, since the average length of the sea trout smolts is usually 3 or 4 inches lower than that of the sea trout from the Waterville system.

Smolt ages.

The distribution of the various smolt classes has been given in Table 2. Four smolt ages, namely, two, three, four, and five-years were observed, the five-year smolts being only of slight importance, forming as they do just over one per cent. of the whole in the maiden fish. Two-year smolts comprised more than half of the total maiden fish examined, and the next most important group, the three-year smolts, were only two-thirds as numerous, forming 37·0 per cent. of the total. The smaller sea trout (i.e. fish in their first post-migration summer) had a greater proportion of old smolts than the larger fish. The two-year smolt class, the youngest group of smolts existing in this river, accounted for 73·3 per cent. of the fish of the second post-migration summer and older age groups, whereas in the first post-migration summer fish the percentage of two-year smolts was only 45·4 (see Table 2). The fact that the younger smolts tend to remain longer feeding in the sea than the older smolt ages is illustrated in Table 3.

Although the results of the present investigation with regard to the age of the smolts are of the same order as those obtained previously (Nall, 1931, and Went and Barker, 1943) there is an increase in the proportion of two-year smolts almost entirely at the expense of the three-year smolts, despite the fact that the proportion of fish in their first post-migration summer is much greater than in the previous investigations. Nevertheless these differences are well within the fluctuations which one might normally expect in a river from time to time.

Age at maturity and number of spawning marks.

In the previously-spawned fish, 29·3 per cent. had spawned for the first time in their first post-migration winter, 57·2 per cent. in their second post-

migration winter, and 13·5 per cent. in their third post-migration winter. There is an apparent discrepancy between the results as obtained from the examination of previously-spawned fish (Table 4) and those obtained by

Table 1. Showing the time and place of the collection of the material from the rod catches in 1943 (see Went and Barker, 1943, page 84 of sketch map of catchment area).

Locality from which data obtained	No. of sets of data collected	Month	No. of sets of data collected
Waterville River	34	Up to and including 30th June	155
Lough Currane	715	July	219
Derriana Lough	37	August	192
Loughnamona	6	September	221
Cloonaghlin Lough	27	1st Oct. to 12th Oct. both days inclusive	58
Loughnahieka	3		
Coppal Lough & River	23		
Total	845	Total	845

Table 2. Proportion of the different smolt ages (maiden fish only) as percentages of each age group.

Age Group	Smolt Age				Total
	2 year	3 year	4 year	5 year	
1st Post Migration Summer	45.4	43.9	8.9	1.8	100.0
2nd, 3rd and 4th Post Migration Summers	73.3	25.7	1.0	-	100.0
Total	56.0	37.0	5.9	1.1	100.0

Table 3. Distribution of the various age groups in each smolt class (as percentages)

Age Group	Smolt Age				Total
	2 year	3 year	4 year	5 year	
1st Post Migration Summer	50.0	73.5	93.5	100.0	62.0
2nd, 3rd and 4th Post Migration Summers	50.0	26.5	6.5	-	38.0
Total	100.0	100.0	100.0	100.0	100.0

Table 4. Age at first spawning in the different smolt classes (as percentages)

Age at first spawning	Smolt Age				Total
	2 year	3 year	4 year	5 year	
1st Post Migration Year	21.5	37.4	33.3	100.0	29.3
2nd Post Migration Year	63.3	50.7	61.9	-	57.2
3rd and 4th Post Migration Years	16.2	11.9	4.8	-	13.5
Total	100.0	100.0	100.0	100.0	100.0

Table 5. Number of spawning marks present in previously spawned fish.

	Number of spawning marks					Total
	1 SM	2 SMs	3 SMs	4 SMs	5 SMs	
Weighted Number	403	119	57	31	12	622
Percentage	64.9	19.1	9.1	5.0	1.9	100.0

Table 6. Proportion of previously spawned fish in the different months (weighted figures used)

Month	Maiden Fish	Previously Spawned Fish
Up to 31st March	10%	90%
April	7%	93%
May	37%	63%
June	74%	26%
July	93%	7%
August	86%	14%
September	85%	15%
Total	77%	23%

Table 7. Calculated mean lengths in inches in fresh water.

Smolt Age	No.	Mean length at end of					Mean Smolt Length
		1st Winter	2nd Winter	3rd Winter	4th Winter	5th Winter	
2	388	3.8	9.0	-	-	-	9.7
3	266	3.1	7.2	9.1	-	-	10.1
4	42	2.5	5.9	8.3	10.0	-	10.4
5	5	2.4	5.4	8.3	10.5	11.8	12.1

Table 8. Calculated mean lengths in inches in the sea.

Age Group	No.	Mean Smolt Length	Mean length at end of			Mean length at Capture
			1st Winter	2nd Winter	3rd Winter	
1st Post Migration Summer	430	9.8	-	-	-	12.0
2nd Post Migration Summer	237	10.5	14.1	-	-	16.0
3rd Post Migration Summer	33	10.1	14.0	17.6	-	20.9
4th Post Migration Summer	1	10.5	13.0	15.2	18.1	19.5
Total	701	9.85	14.0	17.6	18.1	-

Table 9. Calculated mean length in inches at the end of each year of life in the river and sea (for maiden fish only).

Group	Number Examined	River Life						Sea Life		
		Mean length at end of					Mean Smolt Length	Mean length at end of		
		1st Winter	2nd Winter	3rd Winter	4th Winter	5th Winter		1st Winter	2nd Winter	3rd Winter
2+	198	3.5	8.4	-	-	-	9.3	-	-	-
3+	189	3.1	7.4	8.9	-	-	10.1	-	-	-
4+	38	2.5	5.8	8.2	9.9	-	10.4	-	-	-
5+	5	2.4	5.4	8.3	10.5	11.8	12.1	-	-	-
2.1+	171	4.1	9.7	-	-	-	10.5	14.4	-	-
3.1+	64	3.1	6.9	9.4	-	-	10.0	13.2	-	-
4.1+	2	3.2	6.0	8.6	10.3	-	10.8	14.3	-	-
2.2+	18	4.2	9.2	-	-	-	10.1	13.3	16.8	-
3.2+	13	3.2	6.9	9.8	-	-	10.0	14.0	17.7	-
4.2+	2	2.8	6.2	9.0	10.3	-	10.3	13.6	16.6	-
2.3+	1	3.1	9.2	-	-	-	10.5	13.0	15.2	18.1

examination of the maiden fish (Table 3). This discrepancy is more apparent than real, because it is well known that large numbers of whitling (i.e. fish in their first post-migration summer) enter fresh water, sometimes remaining there over the winter, and descend again to the sea without spawning, often accompanied by other sea trout as kelts.

If one assumes that the conditions in the Waterville River are accurately represented by the results given in Tables 3 and 4 then out of every 100 fish spawning, approximately 29 entered the river in their first post-migration summer, i.e. the proportion of whitling to other age groups was 29 to 71. In the entrants to the river in the maiden state the ratio of whitling to other fish was 62 to 38 or say 116 to 71. It follows, therefore, that out of every 116 whitling (fish in their first post-migration summer) only 29 or 25 per cent. spawned in the winter following their first return to fresh water.

It was calculated that 64·9 per cent. of the previously-spawned fish had a single spawning mark on each of their scales, 19·1 per cent. had two marks, 9·1 per cent. three marks, 5·0 per cent. four marks, and 1·9 per cent. five spawning marks (Table 5). It will be noticed that the proportion of previously-spawned fish having a large number of spawning marks on their scales is very much less than in the spring-running sea trout (Went and Barker, 1943, p. 94). Only one fish had spent more than a year recovering from spawning.

Proportion of previously-spawned fish.

As mentioned previously it is impossible to tell the date of entry into the river of an individual fish which has been taken by rod and line. Nevertheless in order to obtain some idea of the fluctuations in the proportion of maiden and mature fish over the season any fish taken on rod and line from the 1st to the 15th day of every month (both days inclusive) was included in the catch for the previous month on the grounds that fresh run fish take a lure more readily than fish some time in fresh water. In Table 6 the results have been given, and it will be seen that in the early part of the season, February to May inclusive, the previously-spawned fish predominated, whereas after that time the maiden fish formed three-quarters or more of the total stock.

Condition co-efficient.

The relationship between the weight and length of a fish, usually called the "condition co-efficient" or "condition factor," is a useful means of assessing the differences in shape which occur over the season or in different districts. As Nall explains (Nall, 1930, p. 263) an accurate comparison cannot be made between fish of different rivers unless they are examined in the same year and month, and after the same interval in fresh water. Obviously with rod-caught fish this ideal test is impossible to achieve. Nevertheless the comparisons are of sufficient interest if we bear in mind that we are dealing, for the most part, with fish which have been up in fresh water for some time before capture.

In the case of sea trout the formula used by workers dealing with British units of weight and length is that devised by Menzies, when working on the sea trout of the River Forth and subsequently adopted by Nall in his intensive studies on Scottish sea trout, namely, $K = W/(L^3 \times 0\ 000427)$ where W is the weight in lbs. and L is length in inches. Another factor, usually called the "condition factor" (according to Corbett's scale) is also sometimes used, particularly by anglers and it is calculated according to the formula C.F. $= 10^5 W/L^3$, where W and L have the same meaning as in the formula mentioned previously. The relationship between the two factors is obviously $K \times 42{\cdot}7 =$ C.F.

In Table 10 the mean condition co-efficients for the various groups have been given, and, even taking into consideration the fact that most of the fish examined had been for some time in fresh water before capture, it seems apparent that sea trout of the Waterville River are slim fish, but it is well known that this character has not affected either their sporting or edible qualities. There is, however, no indication that spawning has affected the average condition of the fish.

Calculated lengths.

As mentioned previously (page 202) the length at the end of every year of life of each fish having suitable scales was calculated in the normal way, i.e. on the assumption that the growth of the fish was strictly proportional to the growth of the scale. Normally the fish having spawning marks on their scales are excluded for obvious reasons. In other words, only maiden fish are deemed to have suitable scales for this purpose. In the previous investigation (Went and Barker, 1943), as in Nall's investigation (Nall, 1931), the scales of a large proportion of previously-spawned fish were also measured, and the appropriate lengths calculated therefrom. It was, of course, realised that the results from such measurements were likely to be somewhat inaccurate, but with the limited number of maiden fish available for examination this could not be avoided. In this investigation the maiden fish sampled, over 700 in number, were sufficiently numerous to give reliable results.

(*a*) *River Life.*—In Table 7 the mean calculated lengths for each smolt class at the end of each year of fresh-water life have been given, and the growth and frequency distribution curves have been given in Fig. 2. It will be seen that the fastest growing parr migrated first.

In the previous work on sea trout of this river (Went and Barker, 1943) it was mentioned that a large proportion of the parr of these fish showed on their scales certain growth rings which correspond with a period of rapid growth in fresh water in the spring prior to migration as a smolt. The transition from river to sea growth is quite well marked in these fish. The difficulty experienced elsewhere with regard to estuarine growth, i.e. growth in the brackish waters of the estuary, is absent, presumably because the Waterville River flows directly into Ballinaskelligs Bay, which opens direct

into the Atlantic Ocean, and in consequence there is no brackish water to provide feeding grounds for the sea trout. It is, therefore, quite simple to calculate the length of the smolts from the scales, and this was done in the

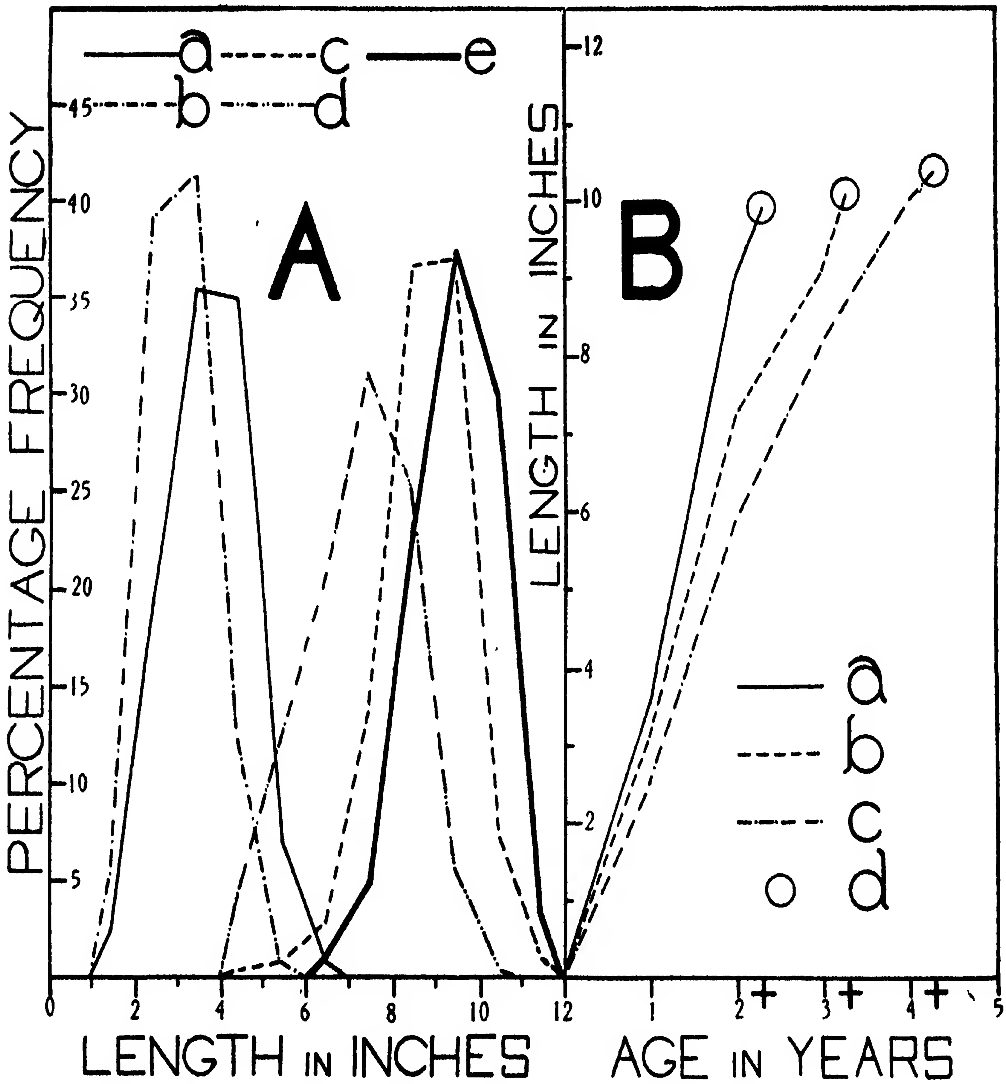

FIG 2.—Growth in fresh water.

A. Distribution curves—
- (*a*) Two-year smolts at one year.
- (*b*) Three-year smolts at one year.
- (*c*) Two-year smolts at two years.
- (*d*) Three-year smolts at two years.
- (*e*) Three-year smolts at three years.

B. Growth curves in the different smolt classes—
- (*a*) Two-year smolt class.
- (*b*) Three-year smolt class.
- (*c*) Four-year smolt class.
- (*d*) Size at migration.

majority of cases. The proportion of fish, which did not show fresh-water growth in the spring prior to migration to the sea as smolts, was exceedingly small, and there was, therefore, no point in separating the fish into two types, as was done in the case of salmon of a number of Irish rivers (see Went, 1938 to 1943). The mean length of the smolt in the various smolt classes increased with a rise in the smolt age (Table 7).

From Table 9 it is obvious that the differentiation in freshwater growth in the various age groups of any one particular smolt class is slight, although the older age groups, i.e. fish in their second and third post-migration summers, have, on the average, a slightly more rapid growth rate in fresh water than fish in their first post-migration summer.

(*b*) *Sea Life.*—Although the number of observations of maiden fish had increased twelvefold, the single specimen of a fish, which returned to fresh water in its fourth post-migration summer described previously (Went and Barker, 1943, page 95) was the only member of its group to be noted in this series of investigations. The calculated lengths at the end of each winter of sea life have been given in Table 8, and it will be seen that in the fish in their second and third post-migration summers the mean lengths at the end of the first sea winter are almost identical. The actual increments made in the sea up to the end of the first sea winter were 3·6 inches and 3·9 inches, respectively, for fish in their second and third post-migration summers (see Table 8), the mean growth rate for all fish being 3·6 inches.

Table 10. Average sizes in the different age groups.

Age Group	Number examined	Length in inches: Minimum	Maximum	Mean	Mean weight in lb.	Mean condition coefficient K	Mean condition factor C.F.
.+	437	9.0	15.5	12.0	0.67	0.90	38.4
.+ 1 SM+	50	13.5	19.75	14.9	1.25	0.82	35.0
.+ 2 SM+	10	15.5	25.0	18.7	2.70	0.86	36.7
.+ 3 SM+	9	17.25	25.0	21.2	4.82	0.90	38.4
.+ 4 SM+	6	19.5	22.8	21.8	4.93	0.87	37.1
.+ 5 SM+	3	20.9	23.2	22.1	6.25	0.90	38.4
.1+	272	11.0	19.25	16.3	1.49	0.84	35.8
.1+ SM+	121	13.5	24.5	19.3	2.54	0.86	36.7
.1+ 2 SM+	73	18.9	25.0	21.7	3.80	0.90	38.4
.1+ 3 SM+	52	18.2	25.5	22.7	4.42	0.94	40.1
.1+ 4 SM+	23	20.9	25.0	23.4	4.78	0.89	38.0
.1+ 5 SM+	6	23.0	26.0	24.6	5.50	0.83	35.4
.1+ SM .1+	1	-	-	24.0	5.75	0.98	41.8
.2+	34	15.75	24.0	20.9	3.96	0.89	38.0
.2+ SM+	41	16.25	25.0	21.7	4.03	0.88	37.6
.2+ 2 SM+	15	21.0	25.5	22.6	4.33	0.86	36.7
2+ 3 SM+	12*	20.5	30.0	26.5	6.34	0.96	40.9
2+ 5 SM+	3	25.0	27.0	25.7	5.75	0.80	34.2
3+	1	-	-	19.5		-	-
Total	1169	9.0	30.0	-	-	0.88	37.6

* Including the largest specimen examined 30 inches long and 10 lb. in weight.

Table 11. Size Distribution (Percentage of weighted numbers). See also Fig. 3.

Age Group	Length in inches *: 9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	30	Total
1st Post Migration Summer	1.1	5.5	12.8	18.9	6.4	0.8	0.2	-	0.1	-	-	-	-	-	-	-	-	-	-	-	45.8
2nd Post Migration Summer	-	-	0.4	0.5	1.2	5.9	7.5	9.1	5.8	2.1	0.7	0.2	-	-	-	-	-	-	-	-	29.4
Total	1.1	5.5	13.3	19.4	8.6	6.7	9.7	10.6	6.1	3.8	2.4	3.3	2.9	2.5	2.1	1.2	0.5	0.1	0.1	0.1	100.0

* Class interval 9 etc. includes all fish having lengths between $8\frac{7}{8}$ and $9\frac{7}{8}$ inches etc.

Average sizes.

The average sizes of the various groups of fish have been given in Table 10. In general it might be said that there is an increase in the mean length and weight after each recovery from spawning, but the annual increment made after the initial spawning is somewhat less than that in the maiden fish. This is not unexpected, as apart from the fact that a kelt has to recover the weight lost as a result of spawning activities, the period of feeding in the sea in any one year is less than in the maiden fish.

Oldest and heaviest fish.

Two fish (0·2 per cent.) were in their eleventh year since hatching, five (0·4 per cent.) in their tenth year, thirty-one (2·6 per cent.) in their ninth year, and sixty-six (5·6 per cent.) in their eighth year. It will be noted that the proportion of old fish is less than that given in the earlier paper, owing to the inclusion of large numbers of late-running sea trout, a large part of which were maiden or immature fish and, therefore, relatively young. The

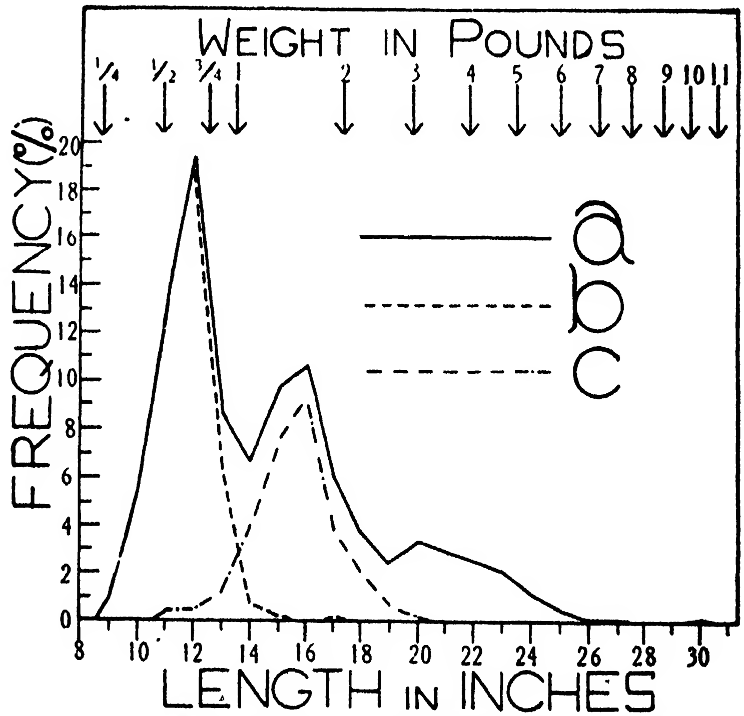

Fig. 3.—Length distribution curves from weighted numbers.

(a) All fish.
(b) Whitling (maiden fish in their first post-migration summer).
(c) Maiden fish in their second post-migration summer.

(Note.—The weight scale drawn up on the assumption that the mean condition coefficient (K) = 0·9.)

heaviest fish was one referred to previously (Went and Barker, 1943, page 96) weighing 10 lbs. and measuring 30 inches.

Size distribution.

The size distribution of the sea trout was estimated from the available data, and the results have been given in Table 11 (see also Fig. 3). It will be seen that there are two peaks in the curve corresponding to the individual curves for the maiden fish in their first and second post-migration summers. In order that the angler should be able to assess the proportion of the various sizes, on the basis of the weight, a scale has been added to Fig. 3 for this purpose, the weight being determined on the assumption that the average condition coefficient was 0·9.

Discussion of results.

In Went and Barker, 1943, p. 97, it was suggested that examination of the late-running sea trout might have given somewhat different results from those obtained by consideration of the spring-running fish. It is now clear, however, that such was not the case when one considers the growth of and size attained by the smolts, and the spring-running fish are obviously merely part of the general stocks of sea trout in this river; not a separate population or entity. The fact that sea trout are to be found in the fresh condition in the early part of the season in the Waterville River is due, in the main, to the early return of the previously-spawned fish. This cannot be explained at present. It is not on account of the necessity to penetrate great distances to the spawning beds, because even to reach the uppermost waters of this river system trout have comparatively short distances to travel.

By combining the material from the net and weir captures with that from the rods a reasonably accurate idea of the stocks of sea trout in the Waterville River can be obtained. The rapid growth in fresh water and the large size attained by the smolts are, more or less, unique as far as sea trout have been investigated to date.

RÉSUMÉ.

(1) New material, consisting of sets of scales and data from sea trout taken by single rod and line in the Waterville River system was obtained and subjected to a preliminary examination. As there was no sign, based on the estimated length of the smolts, of a division of the maiden fish and those maiden fish examined previously (Went and Barker, 1943) into two or more distinct populations (Fig. 1) the new material was added to the material examined previously and the combined material was used thereafter. The possibility of anglers taking quite unwittingly the fry of sea trout has been discussed.

(2) The bulk of the fish migrated as two and three-year smolts the latter being only two-thirds as numerous as the former. There was a tendency for the younger smolts (2 years old) to return to fresh water after a longer interval in the sea than the other smolt classes (Tables 2 and 3).

(3) Just over three-quarters of the total run were estimated to have been maiden fish, and of these nearly 60 per cent. were in their first post-migration summer. As the season progressed the proportion of previously spawned fish, which at first predominated, gradually decreased, and towards the end of the season (from July onwards) less than one-sixth of the total had spawned before (Table 6).

(4) Only 25 per cent. of the fish returning to the river for the first time in their first post-migration summer spawned in the following winter. The remainder may have wintered in fresh water, but did not spawn until, at least, the succeeding winter (Tables 3 and 4).

(5) Previously spawned fish had from one to five spawning marks on their scales. The fish which had only one such mark amounting to about two-thirds of the total, those having two spawning marks were less than one-third as abundant (Table 5).

(6) The lengths at the end of each year of life were calculated for all maiden fish having suitable scales. The fastest growing fish migrated first and there was a rise in average length of the smolts as the smolt classes were ascended (Table 7 and Fig. 2). There appeared to be a slight differentiation in size attained in fresh water in the different age groups (Table 9 and Fig. 1). At the end of the first winter in the sea there was very little difference in mean length in the different age groups (Table 8).

(7) The average sizes in the various groups have been given in Table 10. After each successive spawning the mean length increased, but the increment gradually became smaller (Table 10).

(8) Even allowing for the fact that some fish had been some time in fresh water before capture the average condition coefficient (0 88) suggests that the sea trout of this river are slim fish (Table 10).

(9) In Table 11 the estimated size distribution of the sea trout runs have been given (see also Fig. 3).

Acknowledgments.

I wish to take this opportunity of expressing my thanks to Mr. G. P. Farran, B.A., Chief Inspector of Fisheries, Department of Agriculture, Dublin, for his interest in the progress of the work and for his many helpful suggestions as regards presentation of the material. My grateful thanks are also due to Mr. P. J. Hamilton, Clerk and Inspector to the Waterville Board of Fishery Conservators, for collecting the material and to the numerous visiting anglers to Waterville for their kindness in allowing their catches to be sampled.

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No. 21.

REACTION OF *p*-DIMETHYLAMINOBENZALDEHYDE WITH AROMATIC AMINO COMPOUNDS.

BY A. E. A. WERNER.

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WEIL (1) has pointed out that *p*-dimethylaminobenzaldehyde reacts with aromatic amines to give intense orange precipitates, which he examined in a qualitative way. Sachs and Lewin (2) and Möhlau (3), in discussing the colour of azomethine compounds, refer briefly to compounds which *p*-dimethylaminobenzaldehyde forms with aniline, *p*-toluidine, and *p*-aminodimethylaniline. The present author has described a method for the detection and quantitative estimation of sulphanilamide (4) and sulphapyridine (5) which depends on the formation of an intense yellow colour (or in concentrated solutions an orange precipitate) upon the addition of an acid solution of *p*-dimethylaminobenzaldehyde. Tauber and Laufer (6) have also described a similar colour test for *p*-aminobenzoic acid, depending upon the formation of an orange colour with *p*-dimethylaminobenzaldehyde in glacial acetic acid solution.

In the present communication a systematic investigation of the reaction of *p*-dimethylaminobenzaldehyde with over fifty different amino compounds has been undertaken. The qualitative results obtained may be conveniently summarised thus:—

(*a*) Aromatic compounds react in the presence of mineral acid, provided the $-NH_2$ group is attached directly to the benzene nucleus. Thus aniline reacts, but not benzylamine.

(*b*) Compounds in which a nuclear carboxylic or sulphonic group are present, e.g. *p*-aminobenzoic acid or sulphanilic acid, react immediately in the absence of mineral acid.

(*c*) No reaction occurs with (i) aliphatic amines and amino acids, (ii) N-substituted aromatic amines, (iii) heterocyclic amino compounds, or (iv) amino derivatives of the cycloparaffins, e.g. cyclohexylamine.

The fact that such compounds as methylaniline and acetanilide give no colour when treated with *p*-dimethylaminobenzaldehyde supports the hypothesis suggested by the author and Morris (7), that the chromophoric complex responsible for colour formation is of the Schiff base type, being formed by elimination of water between the aldehydic group and the primary amino group of the aromatic amine. This hypothesis, however, fails to account for the fact that, whereas the Schiff bases normally are feebly coloured substances, the chromophoric complex in question has a much more intense colour; there must be some other factor of paramount importance, which it is the main purpose of the present communication to investigate. With this end in view, the

reaction of *p*-dimethylaminobenzaldehyde with sulphanilamide, aniline, and *p*-aminobenzoic acid has been investigated in greater detail.

When equimolecular amounts of *p*-dimethylaminobenzaldehyde and sulphanilamide were suspended in water, no trace of colour developed, but the addition of a strong mineral acid produced an immediate orange precipitate, a maximum yield being obtained when exactly one equimolecular proportion of acid had been added. This orange precipitate proved to be a salt of the Schiff base with the added acid, so that the reaction may be adequately represented by the following equation:—

$$Me_2N-C_6H_4-CHO + H_2N-C_6H_4-SO_2-NH_2 + HX$$
$$= [Me_2N-C_6H_4-CH=N-C_6H_4-SO_2-NH_2]HX + H_2O$$

The above constitution was proved by analysis of the hydrochloride salt, which was found to contain two molecules of water of crystallisation. This *orange* salt is readily converted by the addition of an equimolecular proportion of alkali or ammonia into a *pale-yellow* compound, which proved to be identical with the Schiff base obtained by Gray, *et al.* (8) on fusing sulphanilamide with *p*-dimethylaminobenzaldehyde. Conversely, the Schiff base so obtained was readily converted into the orange-coloured salt upon the addition of acid. The orange salt obtained by adding *p*-dimethylaminobenzaldehyde to a concentrated solution of aniline hydrochloride behaved in a similar way, and was converted by the addition of alkali or ammonia into an insoluble Schiff base identical with that obtained by Sachs and Lewin (*loc. cit.*) by heating a mixture of aniline and *p*-dimethylaminobenzaldehyde.

This reversible interconversion may be represented thus:—

$$\underset{\text{Orange}}{[Me_2N-C_6H_4-CH=N-C_6H_5]HX} \underset{\text{acid}}{\overset{\text{alkali}}{\rightleftharpoons}} \underset{\text{Pale-Yellow}}{Me_2N-C_6H_4-CH=N-C_6H_5}$$

The insoluble orange "zwitterionic" salt obtained from *p*-aminobenzoic acid, if suspended in water and treated with alkali, goes into solution with a pale-yellow colour, containing the Schiff base,

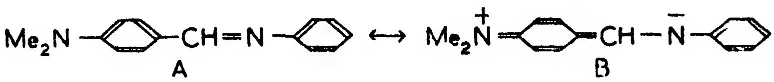
$$Me_2N-C_6H_4-CH = N-C_6H_4-COONa.$$

On standing the pale-yellow colour slowly faded, and glistening crystals separated out, which proved to be *p*-dimethylaminobenzaldehyde, produced by the slow hydrolysis of the Schiff base according to the equation:—

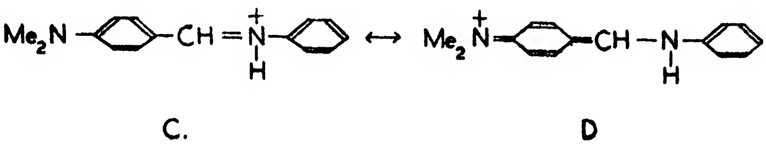
$$Me_2N-C_6H_4-CH=N-C_6H_4-COONa + H_2O = Me_2N-C_6H_4-CHO + H_2N-C_6H_4-COONa$$

In order to understand the marked difference in the intensity of colour between the *orange* salt and the *pale-yellow* free base, it is necessary to consider the intimate molecular structure of the kation of the salt in the light of two factors, which are now recognised as being of paramount importance in connection with the development of colour in organic compounds. These two factors are (i) a conjugated double bond system (cf. Burawoy, 9), and (ii) a resonating system (cf. Brooker *et al.*, 10).

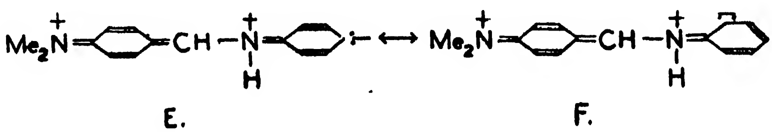
Applying these considerations to the simplest example, namely, the compound formed between *p*-dimethylaminobenzaldehyde and aniline, it is to be noted that the free base can give rise to a resonating system to which the components (A) and (B) contribute:—



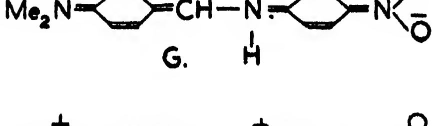
The component (B), however, is much less stable than (A), due to the presence of the negatively charged nitrogen atom, and also the quinonoid ring system[1], and therefore the amount of resonance is limited. Hence the free base has a light colour, due to the presence of the conjugated double bond system in (A). Upon acidification, however, a proton readily adds itself at the negatively charged nitrogen atom, giving the kation of a salt, in which unrestricted resonance is possible. A resonating system results, in which the components (C) and (D)

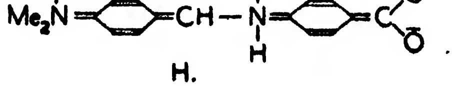


are of primary importance, but to which the components (E) and (F) also contribute:—



The extent to which the components (E) and (F) contribute to the resonating system will be increased, if suitable electron accepting substituents are present in the *ortho* and *para* positions in the aromatic nucleus of the reacting amino compound; thus, in the case of *p*-nitraniline or *p*-aminobenzoic acid, components of the type (G) and (H) will contribute largely to the resonating system.



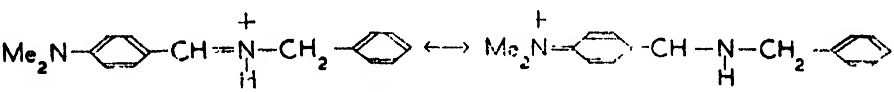


[1] The possibility of resonance between the different Kekulé and other configurations of the benzene ring has a stabilising effect on the benzenoid configuration, which is therefore strongly favoured over the quinonoid configuration.

Furthermore, the more such components contribute to the resonating system, the more perfect the resonance becomes, and hence the more intense the colour of the kation. This deduction has been verified, at least qualitatively, by the experimental findings that the colour given by such aromatic amines as aniline or toluidine is relatively weak in comparison with the colour given by *p*-nitraniline, *p*-aminobenzoic acid, or sulphanilic acid. A quantitative investigation of this aspect of the exact relationship between intensity of colour and chemical structure of the reacting amino compounds has been postponed to a later communication. Passing reference may be made to the fact that the presence of bulky substituents in the *ortho* position to the $-NH_2$ group apparently suppresses the colour intensity. Thus *o*-nitraniline and *sym*-tribromaniline fail to develop any colour upon the addition of *p*-dimethylaminobenzaldehyde.

The essential correctness of the above hypothesis concerning the structure of the kation and the development of colour in these compounds receives additional support from a consideration of the following points:—

(*a*) Aliphatic amino compounds, benzylamine or cyclohexylamine, give no colour upon acidification, although a Schiff base can be prepared according to the fusion method. Now the kation of such compounds can only exhibit resonance to a very limited extent; structures such as (E) and (F) are clearly impossible, and although resonance between components of the type (C) and (D) may be theoretically visualised, e.g. for benzylamine:—



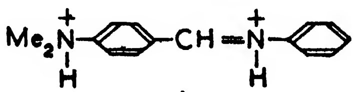
this resonance will be severely damped, if not entirely absent, since the positive charge of the kation must be almost entirely localised on the more basic nitrogen atom. An approximately quantitative idea of the N-N imbalance may be obtained by comparing the ionisation constant of dimethylaniline, namely, $2{\cdot}4 \times 10^{-10}$, with that of benzylamine, namely, $2{\cdot}5 \times 10^{-5}$. (Cyclohexylamine is such a strong base that its aqueous solution readily absorbs carbon dioxide.) Since resonance in the kation is almost entirely suppressed, no colour is developed. There is not even the feeble yellow colour associated with a Schiff base, since "perfect conjugation" (cf. Burawoy, *loc. cit.*) throughout the molecule is absent, conjugation being confined to the three double bonds in the aldehydic portion of the molecule.

(*b*) The colour of the kation depends upon the hydrogen ion concentration. In *dilute* solution an equilibrium is established which may be represented by the following equation:—

$$Me_2N-C_6H_4-CH=N-C_6H_5 + HX \rightleftharpoons [Me_2N-C_6H_4-CH=\overset{+}{N}(H)-C_6H_5]X^-$$

On addition of a mineral acid to an equimolecular mixture of *p*-dimethylaminobenzaldehyde and an aromatic amine there is at first a rapid increase

in colour intensity, which attains a maximum (see experimental part) and then falls off gradually until the solution is finally colourless. The initial increase in colour intensity is due to a shift in the equilibrium in favour of the kation concentration; further addition of acid causes the formation of a doubly charged kation of the structure (I)



I

by the addition of a second proton. A kation of this type is not capable of existing in a resonating state, and is therefore colourless.

(*c*) The colour of the kation is sensitive to the action of heat. On heating an aqueous solution of the kation, the colour intensity gradually diminishes as the temperature increases. This may be attributed to the normal thermal dissociation that all ammonium salts undergo, and in this case involves the following change:

$$[Me_2N-C_6H_4-CH=\overset{+}{\underset{H}{N}}-C_6H_5]X^- \rightleftharpoons Me_2N-C_6H_4-CH=N-C_6H_5 + HX$$

leading to the formation of the feebly coloured Schiff base. On cooling the solution, the colour returns to its original intensity. A similar colour change is also observed on heating the solid salt.

(*d*) Addition of sodium chloride in large excess causes an increase in the colour intensity, whereas addition of ethyl alcohol causes a marked decrease in the colour intensity. Both these salt and solvent effects are associated with a substance in which the following equilibrium with an acid is established:—

$$B + HX \rightleftharpoons B^+H + X^- .$$

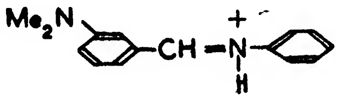
The law of mass action takes the form (11)

$$\frac{[B^+H][X^-]}{[B][HX]} = K\frac{f_B \cdot f_{HX}}{f_{B^+H} \cdot f_{X^-}}$$

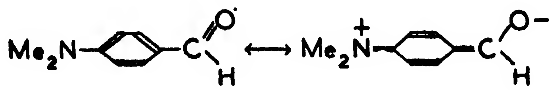
so that the addition of an inert electrolyte, e.g. NaCl, would cause a shift of the equilibrium in favour of the kation B^+H, since it decreases the ionic activity coefficients f_{B^+H} and f_{X^-}. Conversely, the replacement of water by alcohol decreases f_{B^+H} and f_{X^-} thus causing the equilibrium to shift in favour of the free base B, with consequent decrease in colour intensity. It must be noted, however, that the above remarks do not apply to those salts in which sulphonic or carboxylic groups are present in the molecule; in such cases the salt and solvent effects are not so definite nor predictable on theoretical grounds.

(*e*) No colour is obtained when *m*-dimethylaminobenzaldehyde is used in place of the *para* isomeride. There was evidence of Schiff base formation, when the components were heated together on a water-bath, but addition of

a mineral acid did not produce any intense coloration. This lack of colour in the kation having the structure



must be attributed to the fact that resonance is impossible. Furthermore, the fact that Schiff base formation does not readily occur is further evidence of the decreased activity of the carbonyl group in *m*-dimethylaminobenzaldehyde, a fact which has been experimentally shown by Cocker, *et al.* (12), in other reactions of this compound. This reduced reactivity of the carbonyl group is probably due to the fact that, whereas the *meta* isomeride cannot resonate, the *p*-dimethylaminobenzaldehyde itself does show resonance between the structures



EXPERIMENTAL.

Condensation of p-dimethylaminobenzaldehyde with sulphanilamide.—(*a*) Finely powdered *p*-dimethylaminobenzaldehyde (7·5 g.) was intimately mixed with finely powdered sulphanilamide (8·6 g.), and dissolved in hot distilled water (1,000 c.c.). Upon addition of N hydrochloric acid (50 c.c.), the hydrochloride of the Schiff base separated as a copious orange precipitate. Yield = 13·3 g., corresponding to 85 per cent. of the theoretical. Recrystallised from hot water acidulated with hydrochloric acid in the form of dark-red acicular crystals. M.P. 242° C. Analysis (after drying at 105° C.) Found C = 53·20 per cent.; H = 5·31 per cent.; N = 12·38 per cent.; S = 9·55 per cent.; HCl = 10·65 per cent. (argentometrically), 10·70 per cent. (titrimetrically) with N/10NaOH); $C_{15}H_{17}O_2N_3S \cdot HCl$ requires C = 53·0 per cent.; H = 5·30 per cent.; N = 12·36 per cent.; S = 9·40 per cent.; HCl = 10·75 per cent. Loss of weight on drying at 105° C. = 9·4 per cent. $C_{15}H_{17}O_2N_3S \cdot HCl \cdot 2H_2O$ requires 9·6 per cent. By using other mineral acids the following salts were obtained:—Sulphate, M.P. 212° C.; Nitrate, M.P. 200° C.; Oxalate, M.P. 195° C.; Picrate, M.P. 187° C.

(*b*) *p*-dimethylaminobenzaldehyde (7·5 g.) and sulphanilamide (8·6 g.) were dissolved in hot distilled water (1,000 c.c.). Addition of N acetic acid (50 c.c.) caused gradual formation of a lemon yellow precipitate. Yield = 13·0 g., 81 per cent. of the theoretical, M.P. 214° C. (Found N = 13·80 per cent. $C_{15}H_{17}O_2N_3S$ requires N = 13·90 per cent.)

(*c*) *p*-dimethylaminobenzaldehyde (3·8 g.) and sulphanilamide (4·3 g.) were intimately mixed and heated in an oil bath to 140° C. A homogeneous melt is obtained, from which a crystalline solid starts to separate. The solid

mass was maintained at 140° C. for 30 min. The solid residue was extracted with mixture of acetone (20 c.c.) and ethyl alcohol (80 c.c.). Lemon yellow substance remained. Yield = 4·0 g., 54 per cent. of the theoretical. M.P. 212° C. Found N = 13·85 per cent.; S = 10 56 per cent. $C_{15}H_{17}O_2N_3S$ requires N = 13·90 per cent.; S = 10·60 per cent. (Gray *et al.*, *loc. cit.*, give M.P. 229° C.) This compound shows no depression of M.P. when mixed with the compound obtained in (*b*), which must also be the free Schiff base; the expected acetate salt is evidently completely hydrolysed, so that reaction (*b*) provides a better method for obtaining the free base.

Condensation of p-dimethylaminobenzaldehyde with (i) p-aminobenzoic acid, (ii) anthranilic acid, and (iii) sulphanilic acid.—In each case equimolecular proportions of *p*-dimethylaminobenzaldehyde and the respective acids were mixed in distilled water. Rapid deposition of an orange precipitate resulted, and the following compounds were obtained in yields 80–85 per cent. of the theoretical.

(i) Recrystallised from hot water in form of dark-red rosettes. M.P. 245° C. (Found N = 10·23 per cent. $C_{16}H_{16}O_2N_2$ requires N = 10·45 per cent.)

(ii) Recrystallised from hot water as red acicular crystals. M.P. 185° C. (Found N = 10·30 per cent.)

(iii) M.P. 300° C. (Found N = 9·15 per cent. $C_{15}H_{16}O_2N_2S$ requires 9·21 per cent.)

In the case of *p*-aminobenzoic acid the effect of carrying out the condensation in non-aqueous solvents was investigated. Thus in ethyl alcohol and benzene the reaction proceeds very slowly and the yields are much poorer.

Condensation of p-dimethylaminobenzaldehyde with aniline.—(*a*) Finely-powdered *p*-dimethylaminobenzaldehyde (7 5 g.) was suspended in distilled water (400 c.c.) and treated with a solution of aniline (5 c.c.) in N hydrochloric acid (50 c.c.). The solution assumed a yellow colour and rapidly deposited an orange precipitate, which on recrystallisation from N/2 hydrochloric acid yielded red acicular crystals. Yield = 11·5 g., 88 per cent. of the theoretical M.P. 216° C. Found N = 10·65 per cent.; HCl = 13·60 per cent. $C_{15}H_{17}N_2Cl$ requires = 10 71 per cent.; HCl = 13·63 per cent.

(*b*) An intimate mixture of *p*-dimethylaminobenzaldehyde (3·00 g.) and aniline (2 c.c.) was maintained at 120° C. for 30 min. The solid residue was dissolved in hot ethyl alcohol (50 c.c.), from which pale yellow crystals were deposited. M.P. = 99 5° C. Found N = 12·35 per cent. $C_{15}H_{16}N_2$ requires N = 12·50 per cent. Sachs and Lewin (*loc. cit.*) give M.P. 100° C.

Conversion of the salts into the corresponding Schiff bases.—(*a*) The finely-powdered hydrochloride salt derived from sulphanilamide (1·5 g.) was suspended in water and treated with a solution (10 per cent.) of ammonium hydroxide. A pale lemon yellow precipitate was deposited from a colourless solution. Yield = 1·0 g. M.P. 212° C. No depression of M.P. observed when mixed with a specimen of *p*-dimethylaminobenzylidene sulphanilamide prepared by fusion method.

(*b*) On treating the same salt (1·0 g.) suspended in water (100 c.c.) with

N/10 sodium hydroxide (31·5 c.c.) a similar pale yellow precipitate was obtained. Yield = 0·55 g. M.P. 212°–214° C.

(*c*) The hydrochloride salt of *p*-dimethylaminobenzylidene aniline (1·5 g.) was suspended in water and treated with a solution (10 per cent.) of ammonium hydroxide. A lemon yellow precipitate was deposited. Yield = 1·2 g. M.P. 96° C. No depression of M.P. on mixing with a specimen of *p*-dimethylaminobenzylidene aniline prepared by fusion method.

Titration with Caustic Soda.—The following experiment is a typical example of the technique used. Millimolecular proportions of *p*-dimethylaminobenzaldehyde and *p*-aminobenzoic acid were dissolved separately in distilled water (500 c.c.). Aliquot portions (50 c.c.) were mixed; the resulting yellow solution was titrated with N/10 sodium hydroxide, and the number of c.c. required to cause complete decolorisation was noted, a sharp end point being easily obtained. Titre obtained = 10 00 c.c., 9 90 c.c. Theory requires 10 00 c.c., corresponding to the equation:—

$$Me_2N-C_6H_4-CH=\overset{+}{N}H-C_6H_4-CO\bar{O}+NaOH$$
$$\rightarrow H_2O+Me_2N-C_6H_4-CH=N-C_6H_4-COONa$$

Action of Alkali on p-dimethylaminobenzylidene-p-aminobenzoate.—The compound (0·50 g.) was suspended in distilled water (100 c.c.) and treated with N/10 sodium hydroxide (25 c.c.). A yellow solution resulted. On standing 24 hrs. the solution became colourless. Gradual deposition of crystals ensued. Yield = 0·15 g. M.P. 72° C. Found N = 9 25 per cent. $C_9H_{11}NO$ requires 9·40 per cent. There was no depression of M.P. on mixing with an authentic specimen of *p*-dimethylaminobenzaldehyde.

The Effect of pH on Intensity of Colour.—A centimolecular mixture of *p*-dimethylaminobenzaldehyde and sulphanilamide was dissolved in water (1,000 c.c.), and 1 c.c. portions were added to a series of buffered solutions (5 c.c.), ranging in pH from 1 17 to 4·96. The intensity of colour produced in each case was measured visually in a comparator tube. The colour intensity increased gradually up to a maximum at pH 2 05 and then fell off sharply.

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No 22.

THE GAMETOPHYTES OF *PODOCARPUS ANDINUS.*

By W. J. LOOBY and J. DOYLE.

(Plates 6 to 9.)

[Read April 25. Published separately June 8, 1944.]

The simpler species of *Podocarpus* are probably those included in the sub-genus *Stachycarpus*, and of these *P. andinus* is commonly recognized as one of the simplest. There are a number of articles (Coker 1902, Stiles 1912, Sinnott 1913, Buchholz 1941, Tahara 1941) dealing with various developmental phases in the other sections of the genus, notably the Eupodocarpus group. As far as the *pre*-embryonic stages are concerned these papers still leave many points obscure. No complete close study has yet been made of any one species, although in view of the wide differences existing between the various sections it would be desirable to have details, as full as possible, from at least a couple of species from each section for comparison with the anatomical data which are plentifully available. It is still not easy to suggest with a feeling of real probability lines of development within the genus, or fully to distinguish specific and sectional from generic and family characters. For the moment, however, the point of most direct interest here is the fact that there seem to be only two communications referring to *Stachycarpus*. A recent one by Buchholz (1936) deals adequately with the embryogeny proper, but only from the late proembryo onwards, in *P. spicatus, P. ferrugineus*, and *P. usumbarensis*. The other (Sinnott 1913) gives an account of earlier stages in development in *P. spicatus* and *P. ferrugineus*, but the account is in general outline only, details of many of the critical phases being lacking or obscure. Thus on the general development in *Stachycarpus* up to embryogeny there is one short incomplete account, now 30 years old, and in which no reference is made to the most interesting species—*P. andinus*. The only direct reference anywhere found to *P. andinus* is contained in a few comments by Stiles (1912) on its pollen grain while still in the pollen sac. When therefore a specimen of this species with a high degree of fertility became available opportunity was taken to make a close study of as many phases as possible in its life-history.

The present paper deals with gametophyte development up to fertilization. In a second paper it is hoped to cover fertilization and embryogeny stages. Both accounts include some items of detail which may later prove to be of little fundamental importance, but until *Acmopyle* and at least some species of *Dacrydium* have been worked out fully—and in both genera primitive features are to be expected—it seemed better to include these items, as they may help later if only to distinguish characters of less importance from those of greater. A few points also overlap some already briefly described by

Sinnott but, as the overlapping is slight, it was again thought better in the interests of clarity to present a continuous account.

There was available at the same time a fertile specimen of *P. nivalis* whose study yielded some new points in development in a species from the Eupodocarpus group. It is hoped to deal with these in another paper, together with some observations on the podocarps as a whole.

MATERIAL.

Specimens of *P. andinus* are to be found in many estates, but, being ordinarily dioecious, fertile ovules and fruit are comparatively rare. The material used for this study was growing in the National Botanic Gardens, Glasnevin, Dublin. One specimen was noticed to develop young ovulate strobili rather freely, but limited to two large branches on one side. Examination showed that those two branches, at some period when the plant was younger, had been tied with wire for support. The marks of the wire and the swellings above the parts to which the wire had been attached were quite obvious, and this disturbance of conduction was clearly associated with the fertility. About 100 yards away, but with a dense cluster of other trees and shrubs between, grew a staminate plant occasionally bearing a little pollen. This however was ineffective for natural pollination, the ovulate strobili fading and falling off without further development as winter set in. A few attempts at artificial pollination showed however that, if pollinated, the ovules could develop further. On the occasion of a visit in 1938 to the gardens of the Hon Miss Ross at Rostrevor branches were brought back from a specimen there carrying pollen profusely. Surprisingly successful pollination was obtained with this material scattered over the Glasnevin plant, and from the ovules which over-wintered and resumed their development in the spring of 1939 the dates of some of the critical stages were approximately determined. By an accident of extreme good fortune the male tree at Glasnevin produced in 1939 an extraordinarily rich crop of cones, permitting an intense pollination of the young ovules which were also plentiful in that year—and with spectacular results in 1940. The female tree bore hundreds of ripening fruits in dense clusters like rich bunches of grapes. The data obtained from the abundant 1940 collections were supplemented in 1941. The proximity of the Botanic Gardens allowed frequent collection over the critical periods, thus yielding a much closer series of stages than were possible in the study of, say, *Saxegothaea* (Looby and Doyle, 1939), in which case every collection necessitated a journey of about 80 miles.

OBSERVATIONS.

A. *Female gametophyte.* An immature ovule is shown in Fig. 1, no obvious indications of any archesporium being visible at this stage. During the third week of June most ovules are ready for pollination, and have assumed the typical podocarpean form. A young ovule at the average pollination stage

(Fig. 3) shows a small area of poorly differentiated archesporial tissue deep in the nucellus (Fig. 7). This archesporial tissue increases rapidly in size and distinctness, and, in the centre, one cell enlarges as a single gynospore mother cell surrounded by a tapetum (Fig. 8). Comparison between Fig. 3 and Fig. 6, which is a section, four days later, of an ovule at the mother cell stage, shows how rapidly the ovules are growing at this period. Shortly after pollination the micropyle develops typical closing cells for a short length along its distal end. Fig. 2 shows the type. The tapetum is a very conspicuous feature in *P. andinus*. It becomes increasingly obvious (Figs. 5, 6, 8, 9, 11) until the gametophyte begins to expand, when it gradually becomes confused with the disorganizing nucellar cells. It is therefore remarkable that Sinnott records its presence in all the podocarps in which young stages were secured *except* in the species of *Stachycarpus* examined—*P. spicatus* and *P. ferrugineus*. A photograph (his Pl. 8, 39) is given for the latter species at the stage when the functioning gynospore is enlarging. This actually seems to show a layer of cells, different from the nucellar cells, immediately surrounding the spore. As in any preparation the distinctness of the tapetum depends not only on its own degree of development, which shows some variation, but also on both the fixing and the staining used, it would be interesting to revise these two species. If only a very limited number of preparations are available for any stage it could possibly be overlooked.

Three gynospores are formed (Fig. 9), the lower of the two cells resulting from the first division dividing again. These stages are not illustrated, as no peculiar features could be seen in them. Of the three the lowest gives the gametophyte. Exceptions must be very rare, as in no case were more than three gynospores seen, and, in spite of the great number of ovules examined at all stages, in only one ovule were two gametophytes found. The number of spores formed in the conifer groups, three or four, shows little consistent relation, varying often from genus to genus. It is thus perhaps merely a matter of detail, but it is not clear how many typically occur in the two *Stachycarpus* species examined by Sinnott. He *writes* of them, that a "linear tetrad" is formed, but the only figure given, that already referred to for *P. ferrugineus, shows* the developing functioning spore and *two* abortive ones above it, thus suggesting three. The functioning spore becomes vacuolate and enlarges considerably while still uninucleate (Figs. 5, 11). This rather unusual phase, which has not been so far recorded, presents a striking similarity to the corresponding phase in *Saxegothaea* (Looby and Doyle, 1939, Fig. 2), although in *P. andinus* a projecting tip seems absent; nor was any structure later seen like the tiny tent-pole into which it developed in the cellular gametophyte in the former type. But Sinnott's figure, already referred to, of a germinating spore in *P. ferrugineus* almost certainly indicates the occurrence of such a phase in that species also. Development at this period is slow, this stage not being found till well into July, on occasion even not till the end of the month, the whole ovule steadily increasing in size (Fig. 5),

Free nuclear division soon begins, and the gametophyte develops to the stage shown in Fig. 4, in which condition it rests over the winter. Sinnott (1913) does not indicate in what stage his *Stachycarpus* species overwinter, but most probably they behave similarly. The gametophyte of *Saxegothaea* also over-winters as a small, more or less spherical, vacuolate prothallus with parietal nuclei (Looby and Doyle, 1939, Fig. 2a). In *P. andinus* this stage is reached about October, the actual resting stage being a little smaller than that shown in Fig. 4, which is from an ovule in April already wakening from the winter rest. This ovule further shows the very definite development of a wide zone of tissue, which surrounds the nucellus, and which continues above into the micropyle region. This tissue will later form the stone of the ripe seed, the outer tissue forming the flesh, although in both cases further expansion takes place before differentiation begins. The zone appears to be the integument proper, and can already be seen in quite young ovules. It shows with increasing distinctness in the stages illustrated in Figs. 3, 6, 5, 4, and 10. This series, including the still younger stage in Fig. 1, seems to support what is probably the simplest and most obvious interpretation of the fructification in *Podocarpus*. This interpretation considers it simply as an axillary structure bearing a single, attached, inverted, and partly embedded ovule, which is overgrown by the scale tissue, the so-called epimatium.

The exact dates of absolute winter rest were not determined. Increase in size is noticeable as March advances, and stages in typical alveolar formation (Fig. 12) are found by the latter part of April. Unfortunately during this period, and indeed until the gametophyte has become firmly cellular, the whole tissue is extremely delicate, much more so than is even usual at this notoriously difficult stage, and tends to collapse in any fixative. In *Saxegothaea* also the same difficulty was met with in an extreme form. As a result, although the development seems from the material available to be normal, it was difficult to secure stages good enough to photograph. Shortly after the alveoli have met in the centre, and the later typical cell formation has begun, two or three small archegonia can be seen in the upper part. One such is shown in Fig. 13. The actual earliest origin of these is not quite clear. When alveolar formation begins the small vacuolate gametophyte is circular, and obviously there is not room for all the alveoli to advance uniformly to the centre. Many close off early in the typical manner described in a recent paper on *Callitris* (Looby and Doyle, 1940), and are quite small and short, as is shown in the strip of young alveoli in Fig. 12. It is probable that it is two or three of these that actually become the archegonial initials. In most if not all conifers young alveoli, short or long, so function, and in many these can be distinguished from neighbouring vegetative alveoli by their slightly more definite appearance and the obvious lagging of wall formation in them. In the case of *P. andinus* the functioning ones are few and must be very small; they could not be identified till they actually began to show an archegonial appearance some little time after wall formation had begun. At the stage

shown in Fig. 13 outlines of the original alveoli could be still followed throughout the main body of the gametophyte. Sinnott's (1913) only statement on the archegonial origin is that each arises from a "single superficial cell."

At this time also begins a development essentially similar to that described for *Saxegothaea* (Looby and Doyle, 1939). Active periclinal divisions begin in the tissue surrounding the archegonia. An early stage is shown in Fig. 14, taken one section on the ribbon from a young archegonium. Extending in the long axis of the ovule the gametophyte rapidly becomes elliptical, the original centre remaining roughly as the lower focus of the ellipse. A more advanced stage is shown in Figs. 15, 16, the latter illustrating the upper region at a higher magnification. In this way is developed the characteristic cone which extends at maturity below the long archegonia, and which is shown rather coarsely in Fig. 24. The lower tip of the darker area still roughly corresponds to the original centre. The cells in the sub-archegonial area stain particularly heavily, but the cone extends up to the top, and expands well on both sides of the archegonia. The general form is thus essentially similar to that drawn diagrammatically for *Saxegothaea* in Fig. 3c of that paper, although in this form the cells flanking the archegonia tend to stain more heavily than in *P. andinus.* This arrangement of tissue, allowing for minor differences, corresponds to that described by Sinnott (1913) for *Dacrydium* and the species of the *Stachycarpus* group. It seems to be particularly distinct in *P. ferrugineus.* In these cases no indication is given of the nature of its development, but it is presumably like that described above. The cellular differentiation is in general similar to that described for *P. ferrugineus* by Sinnott and for *Saxegothaea* by Looby and Doyle.

As the gametophyte matures the two (or occasionally three) archegonia develop the form shown in Fig. 17. These again closely resemble those described for at least some species of *Dacrydium,* for *Saxegothaea,* and for the species of the *Stachycarpus* group. The jacket cells are not quite so distinct as in *Saxegothaea.* One point however needs further description. In Fig. 18, showing the upper part of the two archegonia in Fig. 17, it is clear that these are still young with the ventral canal nucleus not yet formed. The single-tiered neck can be seen in the left-hand one, and shows that they are already embedded at this stage. Flush, when young (Fig. 16), with the surface of the gametophyte tissue, the latter grows up around them, till at most only a very narrow canal extends from outside down to the necks. For all practical purposes this canal is obliterated by the meeting of the growing tissue, but its position may sometimes be seen clearly enough. This appears in Fig. 19 from a slightly older ovule. Both here and in Fig. 18 the thickening of the outer walls of the neck cells and the small dome-shaped cavity always left just above them are quite distinct. This embedding of the archegonia while still young is without exception the normal occurrence, and has taken place before the pollen tube has reached this area. The tube, at this time, has usually just bored through the nucellus below. The interest of this feature of structure will appear later.

It can also be seen from Fig. 18 that the neck cells, which are in one tier only, may be laterally placed in relation to the archegonial axis, and would be cut obliquely in an ordinary transverse section. This is a frequent feature, but fair transverse views of them are shown in Figs. 21, 22. The number varies from 10 to 15; 13 appear in Fig. 22, and 15 in Fig. 21. The former also shows the sequence of divisions and membrane formation. The original single cell divided into quadrants, each of which could divide into half-quadrants, in each of which again a final division could take place, giving a probable maximum of 16. In Fig. 22, in the lower right-hand quadrant the two half-quadrants have not divided; the next half-quadrant above also failed to divide—thus giving 13 neck cells in all. In Fig. 21, only one half-quadrant did not divide, giving 15. Actually the 16 maximum was never seen, but probably occurs. A further point of interest is shown in Figs. 18, 20. As can be seen from Fig. 18, the archegonial nucleus contains a large nucleolus. In sections stained for ordinary tissues this nucleolus is heavily overstained, so that its structure can only be made out in very lightly stained preparations. In Fig. 20 the large vesicular body is the nucleolus. The small loop to the left is really part of the nuclear membrane, which has contracted in from the outer edge of the nucleus proper, whose limits are shown by the clearer semi-lunar area on the left. Soon after the nucleolus has become really distinct the nucleus proceeds to the ventral canal division. Unfortunately the cytological fate and behaviour of this peculiar nucleolus could not be followed.

The mitosis leading to the formation of the ventral canal and egg nuclei, and which has not previously been recorded for any podocarp, is shown in Figs. 26 and 27. The first of these brings out its position and small size relative to the archegonium; the second, at a higher magnification, the coarsely granular appearance of the neighbouring cytoplasm. There also develops just at this time, in the centre of the upper part of the archegonium, a fairly large zone of more finely granular cytoplasm, into which the egg nucleus retires, leaving the ventral canal nucleus at the outer edge. Fig. 28 is from one of many stages showing this, although the finely granular zone does not come out well in the reproduction. The same appears at a slightly later stage in Fig. 29. Since the mitosis is usually oblique and a little away from the neck, the ventral canal nucleus usually lies to one side of (Figs. 28, 29 and 49), and only occasionally opposite to (Fig. 48), the neck cells. As in so many other conifers, it may be very evanescent, may persist almost up to fertilization, or, very rarely, may enlarge and lie free in the cytoplasm. The egg nucleus enlarges rapidly (Figs. 42, 43, 48, 49), the finely granular cytoplasm forming a neat sheath around it. Two mature archegonia, ready for fertilization, are shown in Fig. 30. In the one on the right the egg nucleus is lying symmetrically, and is cut practically medianly; the large size of the nucleus and the surrounding sheath are very characteristic at this stage. Many scattered vacuoles, large and small, still persist up to fertilization, and, in addition to the fine and coarse granules, larger deeply-staining but irregular-shaped masses, probably protein in nature, occur frequently. These are

however hardly regular enough to be classed as "protein vacuoles." These archegonia resemble closely those described by Sinnott (1913) for *P. ferrugineus* and *P. spicatus*, except that, in these, vacuoles seem a little less numerous. The archegonia of *Saxegothaea*, while similar in general, lack the numerous small vacuoles and apparently also the sheath round the egg-nucleus, the "protein vacuoles" being, on the other hand, more definite.

The gynospore membrane is thick and definite. In Fig. 23, an ovule in rough T.S., the membrane appears as a thick dark line between nucellus and gametophyte, the latter with the characteristic two archegonia. The membrane itself is illustrated in Fig. 25, and shows clearly the inner continuous layer and the outer coarsely fibrillate layer. *Saxegothaea* (Looby and Doyle, 1939, Fig. 3c) possesses a membrane with similar structure. It is possible that the inner continuous membrane may be really double. At the gametophyte apex the membrane thins out rapidly over the archegonial area (Fig. 29).

Considerable changes take place in the meantime in the vegetative tissues of ovule and epimatium. A comparative study in *Podocarpus* of the changes taking place in these tissues up to full maturity has not yet been made, although it would probably yield fruitful results, but, in spite of their interest, a detailed account of *P. andinus* is here omitted except for brief reference to Fig. 10. To facilitate cutting and penetration of fixative this ovule has been sliced down front and rear, but the micropilar region is still intact. The tissues round the epimatial canal have grown and closed it. Within is the micropyle proper. Active division has considerably increased the width of the micropylar part of the integument, but on the outer sides towards its distal part a number of layers of large cells, rather palisade-like in shape, and probably containing resin, have developed. This tissue stops proximally just where the integument fuses with the scale tissue, and distally thins out at the small dark area at the actual micropyle. This dark area is the original group of closing cells shown in Fig. 2. The small-celled part of the integument, sharply marked off from the scale and epimatium tissue, swings completely round and over the nucellus and gametophyte. At the base, to the left in Fig. 10, there can be made out in order from the outside, looser scale tissue, the compact integument tissue, and the nucellar tissue. Actually the scale and integument tissue are separated by a very narrow zone of cells which differ from both. It does not appear in this figure. The loose tissue on the sides of the micropylar part of the integument probably corresponds to the resin tissue described by Gibbs (1912) for *P. ferrugineus* (another of the *Stachycarpus* species), although in this case it ranges right down to the base of the ovule. The narrow zone referred to above probably represents this in *P. andinus*.

B. *Male gametophyte.* This account starts with the shed grain when first found in the micropyle shortly after pollination (Fig. 31). The prothallial cells (or nuclei) are grouped at one side in a small dense clump, which, when lightly stained, can be resolved with high magnification into a number of

separate nuclei, the exact number being difficult to determine at this stage. The nuclear clump in the centre of the upper grain, for example, can be shown to be composed of three nuclei—presumably the tube, body, and stalk, nuclei. The grain rests for about three weeks, sometimes much longer, before germination, which is direct, the outer wall of the grain being retained as in the Pinaceae. As the tube projects the prothallial nuclei enlarge and gradually become free in the cytoplasm, all the nuclei except the body nucleus then passing down the tube, which only penetrates a short distance into the nucellus. It usually branches at the tip. Fig. 33, taken about six weeks after germination, shows a tube and grain cut to one side of the body cell. Eight nuclei are shown, two more appearing on changing the focus—there are thus at least eight prothallial nuclei. Fig. 34 is the same grain from a near-by section on the ribbon to show the body nucleus surrounded by zoned cytoplasm. Growth in this tube is nearly complete, but ordinarily a slight further penetration and a widening of the branches will take place. Fig. 41 is included merely to show the approximate maximum penetration and branching of the tube. The summer growth is probably complete about mid-August. The gametophyte then enters into a state of apparent dormancy, from which it will not waken till the middle of the succeeding May.

This behaviour of the tube and nuclei is again paralleled in *Saxegothaea*—a short summer growth, a long winter rest, the tube tip penetrated only a short distance into the nucellus, the sterile nuclei strung along the tube, and the body nucleus overwintering in the grain. In *Saxegothaea* the prothallial nuclei are, of course, fewer, and lie free in the cytoplasm of the grain before shedding. Sinnott (1913) does not state in what stage the male gametophytes in *P. spicatus* and *P. ferrugineus* overwinter. He merely says that the sterile nuclei precede the body cell down the tube, but from his drawing, Pl. 5, 4, it is practically certain that these species resemble *P. andinus*. *Dacrydium cupressinum*, in which development is spread over two seasons, also shows a similar condition of tube and nuclei during the winter rest (Stiles 1912). It is perhaps of interest that in this paper by Stiles is to be found the only reference in the literature to any gametophytic phase in *P. andinus*. He mentions that up to eight prothallial cells may be found in the grain, which agrees with the maximum number of nuclei seen in the later stages here described. He states further however that, like all other podocarps, the prothallial nuclei in this species come to lie free in the cytoplasm of the grain at the shedding period. Clearly in Fig. 31 it cannot be said that the prothallial nuclei lie obviously free in the cytoplasm in the same sense as they lie free in *Saxegothaea*, for example. This apparent discrepancy raises questions concerning the intimate details of development in the grain while still in the pollen sac, but as these questions do not bear directly on the matter of this paper their discussion can be postponed till certain further data are available. As in other aspects of podocarpean life-history many points still need critical revision in these phases also.

The tube figured in Fig. 32 is from an ovule gathered in the succeeding April, practically nine months later than that in Fig. 33. Although bent, as most of the tubes naturally are, and so only partly in focus, it shows the general stage about ten months after pollination. During the latter part of April and the first part of May the body nucleus enlarges slowly, its associated protoplasm also becoming more compact, but the first signs of awakening activity were not secured till May 29, when the first preparation was got in which the body cell had obviously begun to move from its winter position in the grain (Fig. 35). Development at first is not rapid, so that in the first week of June many stages were secured like that in Fig. 36, which shows a cluster of tubes, in two of which the body cell has moved well down. It also seems clear, in spite of the branching of the tube at the tip, that all, certainly most, of the sterile nuclei proceed down one branch, usually the most advanced branch, and that the body cell follows in the same path; in later stages a number of sterile nuclei are always found in front of it. The cytoplasm of the body cell is still rather ill-defined from that of the rest of the pollen tube.

Subsequent development is more rapid, and fertilization is commonly effected within ten days of the body cell reaching below the level of the nucellar surface. Special attention was given to this subsequent history of the tube and the body cell. In the paper on *Saxegothaea* (Looby and Doyle, 1939) a short survey was given (pp. 110–111) of our knowledge of the male cells in the podocarps as a whole, following the demonstration of the occurrence in that plant of two equal male cells. With the exception of *Microcachrys*, which in any case needs revision, the podocarps have been taken to be characterized by an extreme inequality of the male cells, the smaller of which, in *Podocarpus* proper, is said to be represented merely by a nucleus, partly or wholly extruded from the cytoplasm of the functioning cell. The survey showed that this concept, while probably quite correct, is actually based on such meagre evidence that a critic might readily reject it. In most cases only fragmentary data are given; in no case is a complete history of the tube from germination to fertilization available, and the only really cogent evidence, that supplied by Coker in 1902, was derived from the examination of only two pollen tubes. To secure stages in the male gametophyte of *P. andinus* was thus advisable, especially as the species is one of the simplest in the genus.

As the tube begins to grow more rapidly it penetrates the lower half of the nucellus, in which the cells are compact and rather densely filled with starch grains. Here the tube is narrow, as if certain difficulties in penetration existed, and the whole body cell becomes drawn out. Staining at this stage is also difficult, the dense cytoplasm colouring so intensely as to obscure the nucleus. A typical view, at a low magnification, is given in Fig. 37, merely to show the condition. In this the small upper darker area of the dense mass in the tube is the body nucleus, which is actually in the prophase of its division, although it proved impossible to record this photographically at any magnification. A number of nuclei, also not shown, are embedded in the

slightly lighter protoplasm in front. As the tip gets through the nucellus the whole tube seems freer to widen and expand a little, the body cell becoming more elliptical and normal in shape. At this stage the body nucleus divides. The difficulty of contrast staining still continues, however, so that the only mitosis which could be clearly recorded (Fig. 38) was in a tube with a marked bend just at this level. This mitosis in the body cell, recorded here for the first time in any podocarp, occurs at the upper part of the body cell. The lower of the two nuclei formed moves slowly to the centre of the cytoplasm, and becomes gradually larger than the other, which remains in position above. By this time the contents of the tube have reached at least the level of the lower surface of the nucellus. Figs. 39 and 40 show two successive sections of the same tube. It is thus already clear at this stage that an extreme inequality of the male cells will result. Fig. 39 shows the principal male nucleus lying in the main body of the cytoplasm; the second male nucleus, which will not function, is apparently almost extruded already from the main cytoplasm (Fig. 40), although it is doubtful if extrusion ever really occurs.

Before, however, commenting on the various stages reproduced to illustrate the maturing of the male cells a word is necessary on the growth of the pollen tube. Sinnott (1913) definitely states for *P. spicatus* and *P. ferrugineus* that, while still young, the pollen tubes lie over the archegonia, which are still almost flush with the surface of the prothallus. The surrounding gametophyte tissue then grows up round the pollen tubes, enclosing them in a deep cavity, at the bottom of which the neck cells lie. The result is figured for *P. ferrugineus* in his Pl. 5, 2. He further states that any archegonium not reached in time by a pollen tube will also become embedded, and cannot then subsequently be fertilized. In view of what can readily be followed in *P. andinus*, Sinnott's account is almost certainly the result of incorrect interpretation from too few preparations. In *P. andinus* the tube burrows between gametophyte and nucellus till it comes to lie over the thin area of the gynospore membrane (Fig. 29) above the position of the archegonia already deeply embedded. It bores through the membrane and burrows down approximately through the canal left above the necks by the upgrowth of the gametophyte tissue. In Fig. 42, which includes the full length of the archegonia, the tube, indicated really by its contents, can be seen pushing into the canal above. Fig. 43 shows the same tube and the upper part of the archegonia at a higher magnification. Fig. 44, from the next section on the same ribbon, shows clearly the boring tip of the tube. Stages in the further penetration to the necks appear in Figs. 45, 46, 47. On reaching the neck the end of the tube spreads out over the top of the archegonium, to produce the effect shown in Fig. 48. Although the tube contents in this preparation are badly contracted it is included here, as it shows this splay effect so well, but the same appearance is also clearly seen in Figs. 49, 50, 51, 52, 55 and 56. The cells over the top of the archegonium are compressed and presumably digested away till the neck cells finally remain swung from the sides by a membrane only. From Sinnott's

rather diagrammatic drawing, which corresponds in *P. ferrugineus* to this group of figures, we are convinced that the behaviour in this species, and presumably also in *P. spicatus*, would prove to be essentially similar to that in *P. andinus*, if a suitable series of stages were available.

Further, this behaviour in *P. andinus* definitely helps to clarify the corresponding stage in *Saxegothaea* and *Phyllocladus*. In the former, when ready for fertilization, the archegonial necks are sunk, but only in an open shallow pit. The figure, Pl. 2, 10, in the paper by Looby and Doyle (1939) shows a fairly common condition in *Saxegothaea*, the presence of only one effective pollen tube, so that an undisturbed archegonium can be found beside one about to be fertilized. Obviously the pollen tube has grown down, clearing away the lateral cells and pressing the resistant archegonial (?) membrane in front of it with the neck cells suspended in the centre. This arrangement of the neck cells at this stage must have been characteristic of the primitive podocarpean type. It is shown in species of *Dacrydium* (Stiles, 1911) and *Phyllocladus*, in which latter genus it was first recorded by Young (1910). Young was of the opinion that the condition was produced not only by pollen tube pressure, but also by upgrowth of prothallial tissue round the tube. Every figure of these stages published in Young's paper shows, however, an archegonium reached by a pollen tube, no comparative control in the form of an archegonium without a tube, such as is available in *Saxegothaea*, being discussed. As Young's material, though apparently very fertile, was not plentiful, and as the archegonia, when first reached by a pollen tube, are also sunk in a shallow open pit, it seems reasonable to infer that the behaviour in these two types is essentially similar and differs from that in the *Stachycarpus* species only in the shallower position of the archegonia.

When the tube begins to bore into the gametophyte tissue the male cell complex (Fig. 43) is usually only a little further advanced than the stage shown in Figs. 39, 40. The functioning nucleus is clearly seen in the centre, the smaller, not quite in focus, above. In every case in which fixation and staining were satisfactory, this small nucleus could be seen to be still embedded in the cytoplasm. This point will be returned to shortly. A clump of sterile nuclei, which always lies in front of the male cells, can be seen in Fig. 44. In the next figure, Fig. 45, the smaller male nucleus is barely indicated above, but there is seen below a zone of looser cytoplasm, which occupies the anterior part of the tube, and from which the male cell cytoplasm seems fairly well delimited. Actually, however, the small dark specks, the sterile nuclei, below the visible male nucleus are themselves embedded in cytoplasm, from which only later will the male cell proper be separated. In Fig. 46 the male cells are older, the nucleus of the larger one now showing the characteristic nucleolus—the dense dark area. The pollen tube in Fig. 47, though a poor one, is included, as it shows the smaller nucleus in front of the other. This is fairly common (10 per cent. of cases?), but its origin in relation to the body cell division was not determined. Maturing male cells are shown in Figs. 49, 50. Here the

anterior cytoplasm, shown in Fig. 45, has spread out over the archegonial necks; the zone of cytoplasm containing the sterile nuclei appears a little distinct from the male cells; and the cytoplasm of the functioning male cell has become more clearly delimited. The male cells are still further advanced in Fig. 51. The zone in which the sterile nuclei are embedded is definitely distinct, and in fixation tends to separate from the male cells. The delimitation above between the smaller and larger male cells is still a little indefinite.

P. andinus seems definitely to form two male *cells*, although these are very unequal in size. The real nature of the smaller male cell sometimes can be seen very clearly, as in the stages illustrated in Figs. 57, 58, 59, and 52. The first of these figures shows a definite delimitation of an area of cytoplasm surrounding the smaller nucleus above. The other nucleus was only partly included in this section, and does not show in this plane of focus. It is unusual to notice so clear a separation in so young a stage, or to find so relatively large an amount of cytoplasm associated with this smaller nucleus. Fig. 58 is more typical of the advanced stages. The functioning male cell is almost mature, as shown by the extraordinary large and dense nucleolus and the indications of an outer membrane. The narrow belt of cytoplasm shows distinctly round the smaller nucleus, which also contains a similar nucleolus. In this case the area containing the sterile nuclei is overlying the lower part of the larger cell, and obscures its lower boundary. In Fig. 52 this zone of protoplasm has contracted away, leaving isolated the functioning male cell, which is just mature. In this case the second male cell lay in the same position as that shown in Fig. 47. Its nucleus is in a neighbouring section, but here there appears a thin projecting vesicle at the lower right corner, which is the thin layer of cytoplasm which surrounded it as in Fig. 58. The smaller complete male cell is also clear in Fig. 59, from a stage immediately before fertilization. The large male cell is again thrown out of focus in order to emphasise the line of demarcation between the two. Admittedly in cases where fixation was poor the presence of a protoplasmic layer round the second male nucleus was not so certain, but it is considered probable that in all cases two very unequal male *cells* are present.

A striking feature in the male cells, particularly at maturity, is the enormous nucleolus. As the plate figures show, it colours intensely, but a complex vesicular structure (Fig. 60) can be seen when very lightly stained. In this case it is cut tangentially, as the vesicular appearance is then a little more definite for photographic record. The nucleolus of the smaller male cell is similar.

Development proceeds in a very uniform manner in most cases, irregularities or departures from the described sequence being few. Thus in one case the male cells developed large nucleoli while still in the nucellus (Figs. 53, 54). This was from one of the successful pollinations with Rostrevor pollen, which may perhaps mature more rapidly. On the other hand, the male cell in Fig. 29, from Glasnevin pollen, is also somewhat advanced for a tube in

this position. The smaller male cell in this case was not in the plane of this section. Its absence rather over-emphasizes the distinctness of this functioning one. There occurred, however, one feature which could not be interpreted with certainty. The tube in Fig. 55 seems to contain two subequal male cells in close contact, the nucleus of the upper being a little indistinct. The preceding section of the ribbon, not here reproduced, shows a small area of the lower part and a fairly definite circular tangential section of the upper. The section succeeding Fig. 55 on the ribbon is shown in Fig. 56. The main body here is a section of the larger lower area in Fig. 55 cut tangentially to the nucleus, but at the lower right corner lies a body which shows every appearance of being a typical smaller male nucleus in a forward position. At least three obvious interpretations could be put forward, including the possibility of this being a reversionary development of two equal male cells, but any suggestion based merely on this single equivocal occurrence is really valueless.

Fertilization is now due to take place, and will be described, with the later stages, in a succeeding paper.

Summary.

The female gametophyte, and the male gametophyte from the lodgment of the pollen grains in the micropyle, are described, for *Podocarpus andinus*, up to the fertilization stage. In form, the mature female gametophyte closely resembles that already described for *Saxegothaea* and certain species of the *Stachycarpus* group. Earlier stages are also similar to those in *Saxegothaea*. Both show a single mother cell giving three gynospores, a distinct tapetum, marked increase in size and vacuolization of the functioning spore before the first nuclear division, overwintering as a small circular vacuolate prothallus, and development around the young archegonia of a meristematic cone, which forms most of the mature gametophyte. *P. andinus* only differs in the deep embedding of the archegonia while still young.

The pollen tube penetrates only a short distance into the nucellus, and overwinters with up to ten nuclei strung along the tube—the stalk and body nuclei and up to eight prothallial nuclei—leaving the body nucleus with associated cytoplasm resting in the grain, from which position it does not move till about eight months later. Except for the smaller number of sterile nuclei *Saxegothaea* shows a similar resting phase.

Special attention was given to the later development of the pollen tube and the body cell to clarify the nature of the male gametes in one, at least, of the simpler species of *Podocarpus*. The tube bores down through the overlying tissue to reach a deep-seated archegonium, over the top of which it splays out. The origin of the isolated position of the neck cells, recorded for many podocarps, is explained. Division in the body cell is recorded for the first time in any podocarp. A series of stages in the organization of the male gametes is described. There develop two unequal male *cells*; the smaller may be very

definitely delimited from the larger functioning one. Only in this feature of the inequality of the male cells do the gametophytes of *P. andinus*, in general, differ from those of *Saxegothaea*.

EXPLANATION OF PLATES.

PLATE 6.

1. L.S. very young ovule and subtending bract. June 6. × 45.
2. L.S. micropyle to show position and nature of closing cells. June 29. × 150.
3. L.S. ovule at pollination to show general form and size. June 19. × 45.
4. L.S. ovule, partly cut away, to show circular vacuolate gametophyte. April 19. × 20.
5. L.S. ovule at stage of vacuolate uninucleate gametophyte with good tapetum. July 30. × 45.
6. L.S. ovule at stage of gynospore mother cell; tapetum distinct. June 23. × 45.
7. Part of 3 at higher magnification to show young archesporial tissue deep in nucellus. June 19. × 150.
8. Gynospore mother cell with surrounding tapetum and nucellar cells. June 23. × 300.
9. Row of three gynospores with surrounding tapetum. June 29. × 300.
10. L.S. older ovule sliced away on two faces to show developments in integument; also cellular gametophyte with young archegonia. May 28. × 20.

PLATE 7.

11. Uninucleate vacuolate gemetophyte, somewhat contracted, with surrounding tissue. July 30. × 140.
12. Early alveolar formation. April 23. × 300.
13. Very young archegonium with surrounding tissue, recently derived from alveoli. May 12. × 140.
14. Initiation of active periclinal divisions in tissue surrounding the young archegonia; traces of recent alveolar formation still visible. May 12. × 140.
15. Young gametophyte, with developing archegonia; explanation in text. May 28. × 45.
16. Archegonial area of 15 at higher magnification. May 28. × 65.
17. Complete maturing archegonia just before formation of ventral canal nucleus. June 10. × 45.
18. Upper portions of same showing nucleus, necks and overgrowth of gametophyte tissue. June 10. × 140.
19. Area just above the neck of an older archegonium to show the narrow canal formed by the overgrowth of the gametophyte tissue. June 19. × 300.
20. The peculiar nucleolus of the archegonial nucleus at stage of Fig. 18. June 10. × 800.
21. To show 15 neck cells. June 10. × 140.
22. To show method of division of primary neck cells. June 10. × 600.
23. T.S. of part of gametophyte showing two archegonia with jacket-cells; the surrounding nucellar tissue and the thick gynospore membrane. June 10. × 45.

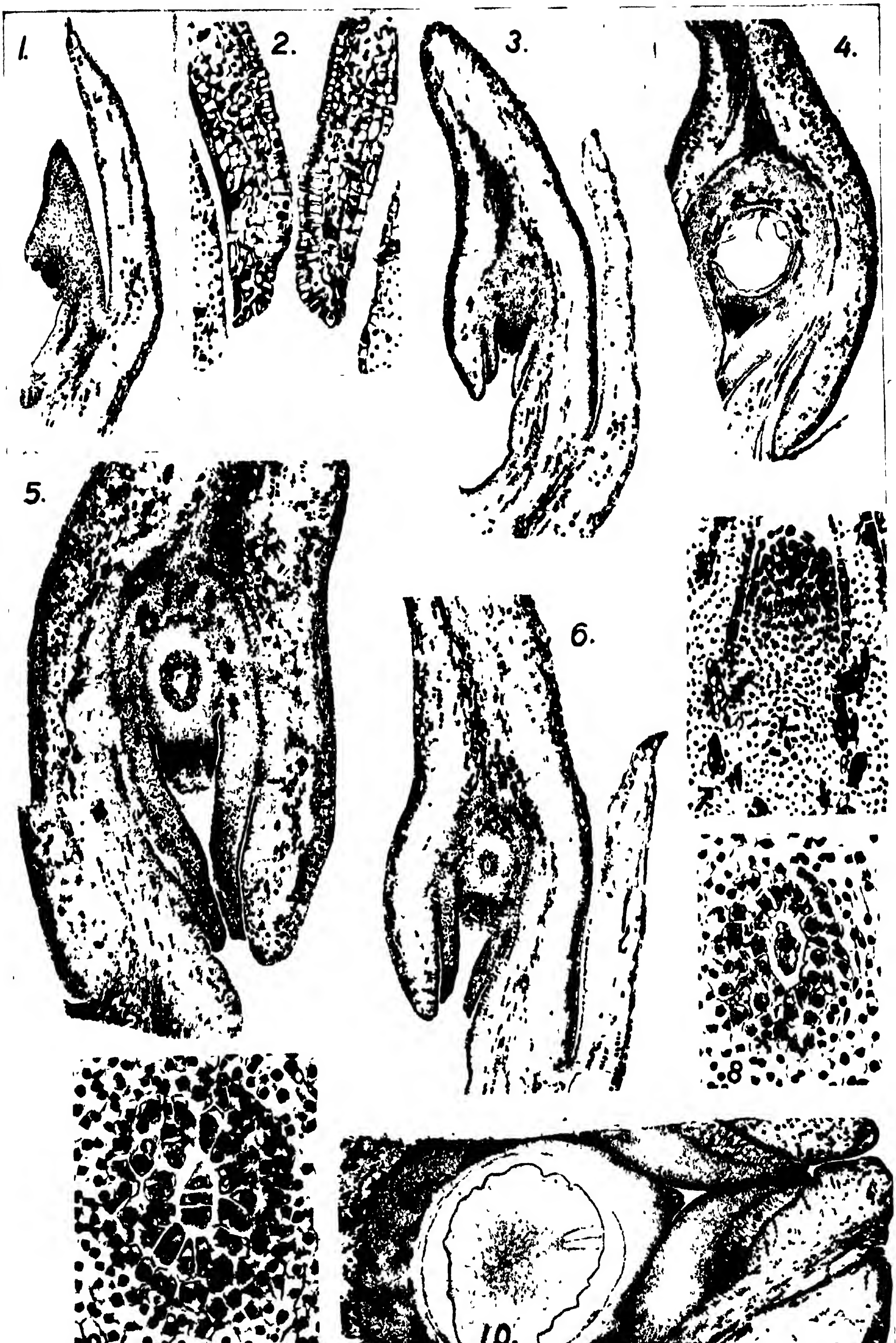
24. L.S. complete gametophyte to show the cone of tissue stretching below the archegonia to position of the orginal centre. June 10. × 20.
25. Gynospore membrane to show inner continuous layer and outer fibrillate layer. June 10. × 1600.

PLATE 8.

26. Division cutting off ventral canal nucleus to show position and size relative to archegonium. June 14. × 140.
27. Same at higher magnification showing the coarsely granulate cytoplasm. June 14. × 300.
28. Egg nucleus, shortly after the division, retiring into the central more finely granular zone of cytoplasm; ventral canal nucleus still visible. June 14. × 300.
29. Upper portion of archegonium at slightly later stage to show egg nucleus, now within the finely granular zone; ventral canal nucleus; the pollen tube, containing a rather advanced male cell, just before its penetration of the thin area of gynospore membrane above position of deeply sunken archegonia. June 13. × 140.
30. Two mature archegonia; on right egg nucleus, with finely granular sheath, is cut medianly; pollen tubes down to necks in each case; tube on left cut tangentially but includes a mature male cell. June 17. × 140.
31. Pollen grains in micropyle. June 27. × 300.
32. Resting winter stage of pollen tube, the sterile nuclei strung along the tube, the body-cell left behind in the grain. April 23. × 300.
33 Pollen grain and tube shortly after germination, 8 sterile nuclei passing down, body cell in next section (33). July 30. × 600.
34 Young body cell in same grain, as 33. July 30. × 600.
35. Body cell just moving from resting position in the grain. May 29. × 300.
36. Cluster of pollen tubes in two of which the body cell has moved well down. June 6. × 140.
37. Pollen tube just through the nucellus, detailed description in text. June 13. × 150.
38. Division of body cell nucleus. June 13. × 600.
39 and 40 The two male nuclei shortly after the division of the body nucleus. In 39 the functioning male nucleus and in 40 the non-functioning one; body cell cytoplasm poorly differentiated from the rest of the protoplasm of the tube, especially anteriorly. June 13. × 300.
41. To show branching of pollen tube; with 32, shows maximum development of tube at resting stage. April 23. × 140.

PLATE 9.

42. Full length archegonia almost mature; pollen tube with young male cells just penetrating gametophytic canal above. June 13. × 45.
43. Tube boring down to neck; functional male cell still quite young; second male nucleus just visible above the larger functional one. June 13. × 150.
44. The boring tip of the pollen tube; sterile nuclei visible; from same tube as 43 but a section further on. June 13. × 300.
45. Another tube just down to level of archegonial necks; male cell still young; upper non-functional male nucleus barely indicated above; male cytoplasm not yet fully marked out from rest; sterile nuclei bunched in what appears to be the lower part of the male cell cyptoplasm. June 13. × 300.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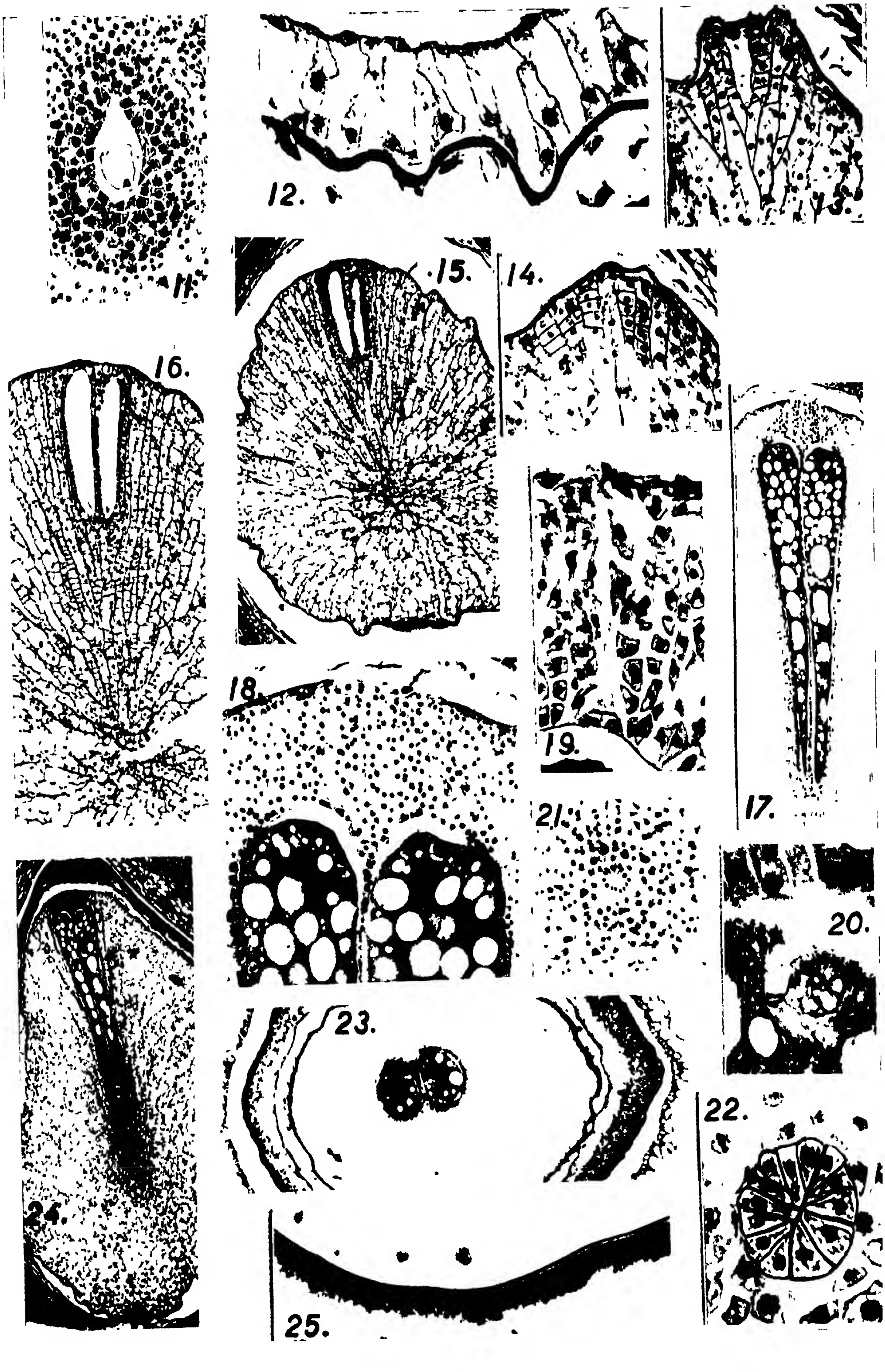
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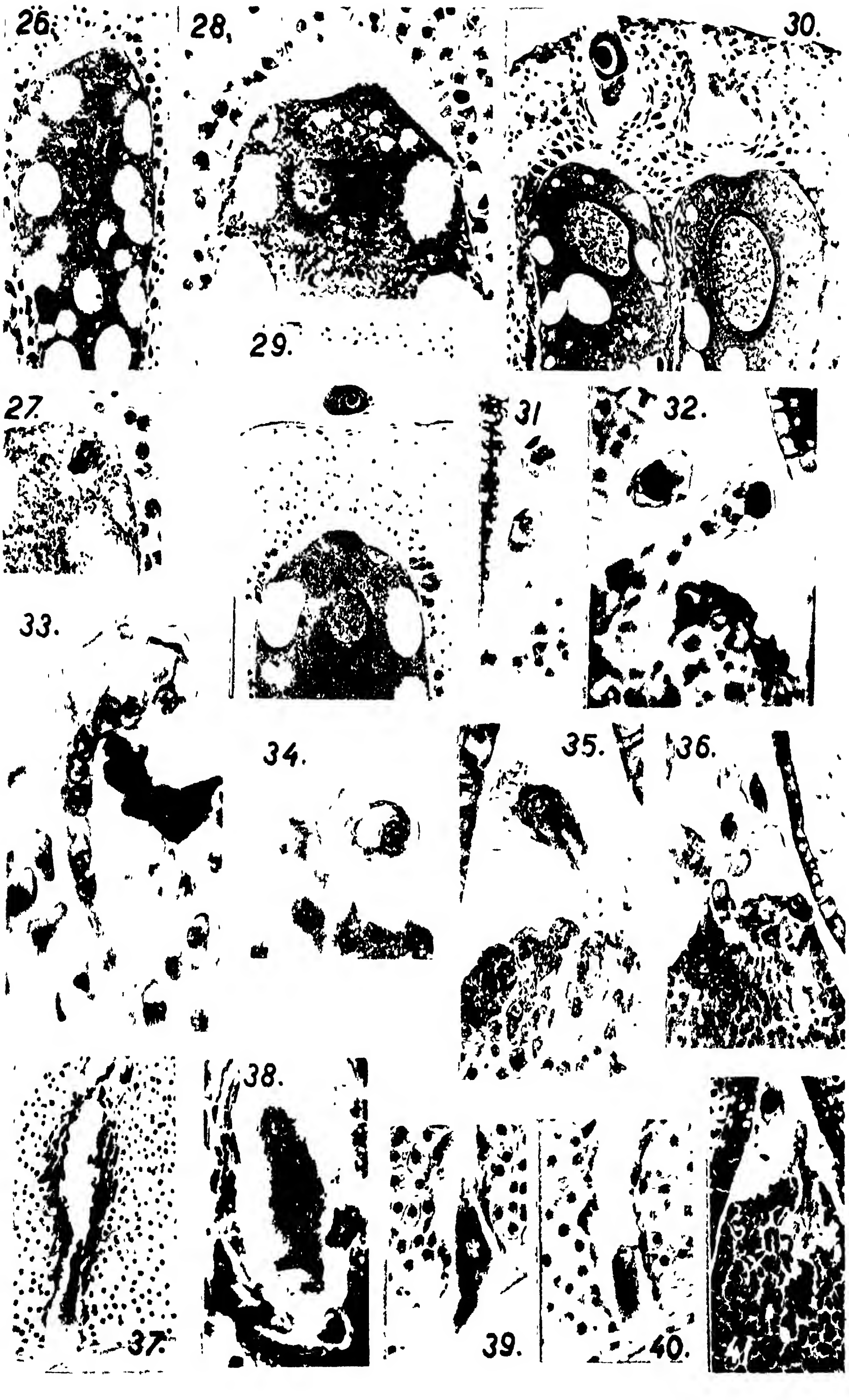
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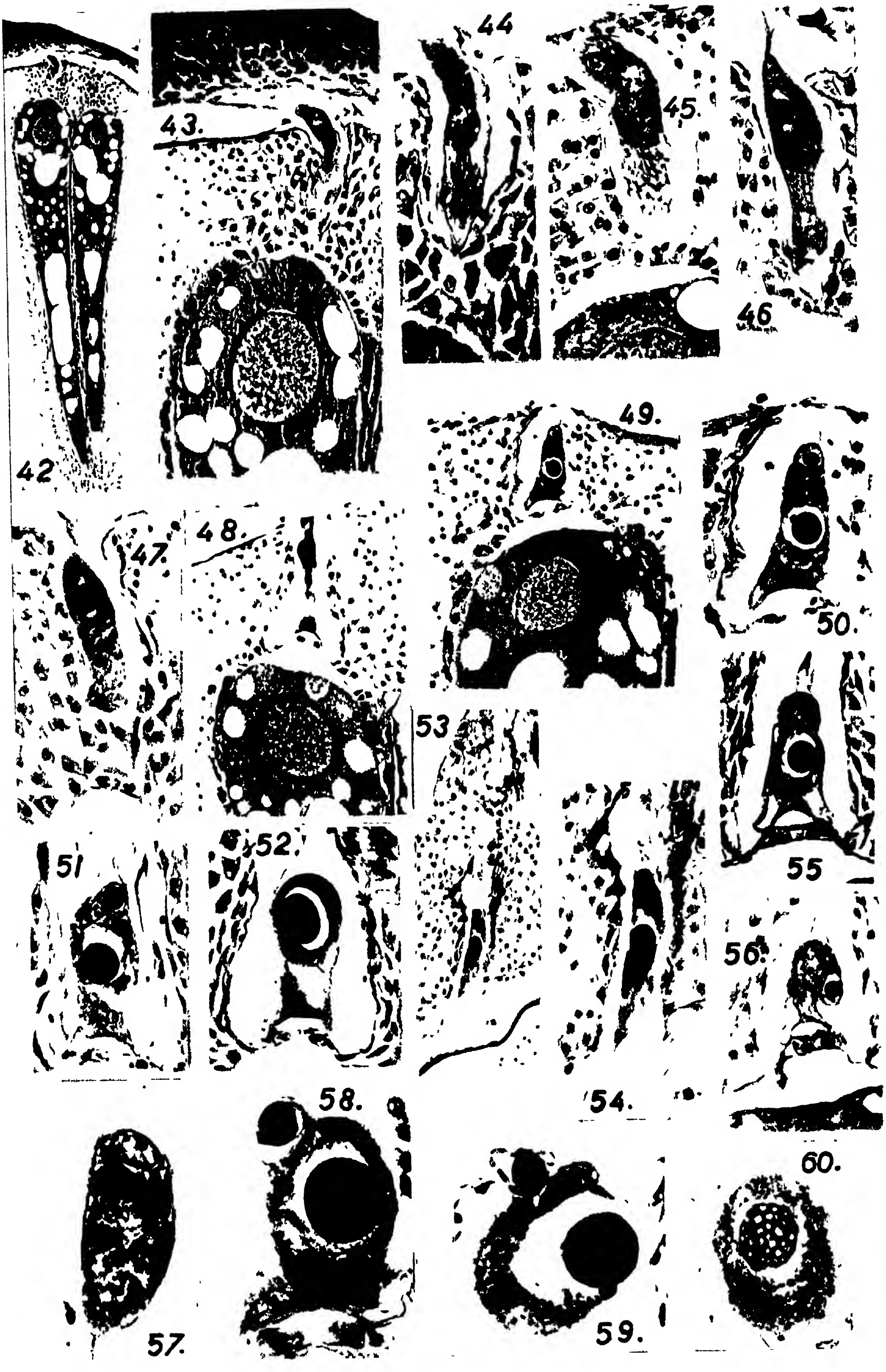
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46. Slightly older male cell; nucleolus of functioning nucleus enlarging; sterile nuclei, probably not visible in reproduction, bunched as in 45; non-functioning male above. June 13. × 300.
47. Similar stage to show non-functioning male nucleus embedded in cytoplasm in front of the functioning one. June 13. × 300.
48. To show how the end of the pollen tube splays out over the top of the archegonium to leave neck cells attached by a membrane to the side; tube contents poor, but egg nucleus, surrounded by finely granular sheath, and ventral canal nucleus clear. June 16. × 150.
49. Maturing male cells over archegonium; ventral canal nucleus still visible. June 13. × 150.
50. Male cells at a higher magnification, cytoplasm of functioning one becoming more distinctly limited from zone below containing the sterile nuclei. June 13. × 300.
51. Male cells almost mature showing the enormous nucleoli; the smaller nucleus sheathed in protoplasm; the functioning cell clearly distinct below from the zone containing the sterile nuclei, several of which are visible. June 13. × 300.
52. Male cell mature. In this case the smaller male cell (which appears in another section) was below; here its thin protoplasmic sheath is shown at the lower right corner of the male cell. June 17. × 300.
53. Male cells with large nucleoli formed while still in nucellus. June 14. × 150.
54. Same at higher magnification. June 14. × 300.
55 and 56. Unusual male cell complex. See text for explanation. June 15. × 300.
57. Young male cells, nucleus of larger only faintly visible in this section, which shows the definite delimitation of the smaller as a distinct male cell. June 13. × 600.
58. Mature male cells, the smaller with its distinct cytoplasmic sheath; the delimitation of the larger and the membrane surrounding it thrown out of focus a little to secure the smaller. June 13. × 600.
59. Similar stage from a male cell complex immediately before fertilization, functioning male cell considerably out of focus in this case. June 16. × 600.
60. Nucleolus of a functioning male cell cut tangentially to show its structure. June 13. × 600.

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No. 23.

REPORT OF THE RADIUM COMMITTEE FOR THE YEAR 1943.

[Read May 23. Published separately June 8, 1944.]

647 tubes containing 5077 millicuries were issued in 1943, as compared with 1109 tubes containing 6648 millicuries in 1942.

Mr. W. M. Hynes was elected to the Radium Exhibition for 1943–44.

Reports from users record the treatment of the following cases with radon or radium element during 1943:—

	Malignant.	Non-malignant.	Total.
Dr. O. Murphy, St. Vincent's Hospital, Dublin ...	80	82	162
Dr. O. Chance, Richmond, Sir Patrick Dun's, Rotunda, and Coombe Hospitals, Dublin ...	24	5	29
Dr. Bethel Solomons	—	3	3
St. Anne's Hospital, Dublin	136	—	136
Totals,	240	90	330
Totals for the same users in 1942	234	108	342

The reports show that for cases of malignant disease first seen during each of the years 1941, 1942, and 1943, the following methods of treatment were adopted:—

	1941.	1942.	1943.
Cases seen	1204	928	964
Cases treated, Total	1015	730	779
" " by Surgery alone	176	54	43
" " by Radium alone	222	184	187
" " by X-Rays alone	457	406	464
" " by Surgery and Radium	30	17	13
" " by Surgery and X-Rays	68	26	32
" " by Radium and X-Rays	60	41	35
" " by Surgery, Radium, and X-Rays ...	2	2	5

As there is no appreciable decline in the number of cases receiving radium treatment in 1943, as compared with 1942, it appears that the decreased use of radon must be attributed to an increase in the use of radium element.

Dr. Murphy reports the radiological treatment of the following cases:—

Malignant.—Carcinoma of lip, 6; ear, 2; floor of mouth and tongue, 5; breast (secondaries), 4; bladder, 1; uterine body, 2; uterine cervix, 12; skin surface, 14. Rodent ulcers, 36. Total 82, of which 80 were treated with radon or radium element.

Non-malignant.—T.B. Peritonitis, 8; Uterine hæmorrhage, 4; Angiomata, 18; Lupus vulgaris, 2; Lupus erythmatosis, 2; Keloids, 3; Warts, 46. Total 82; all treated with radon or radium element.

Dr. Chance reports the radiological treatment of the following cases:—

Malignant.—Carcinoma of skin, 21; lip, 20; tongue 11; cheek, 2; palate, 2; tonsil, 2; alveolus, 3; larynx (extrinsic), 2; pharynx, 1; glands of neck, 4; breast (*a*) operable, 16, (*b*) inoperable, 3, (*c*) post-operative recurrence, 3; cervix, 3; fallopian tube, 1; vulva, 1; bladder, 1; lung, 1; rectum, 2; pituitary gland, 1; spinal cord, 1; post-cricoid carcinoma, 3. Sarcoma (various), 5. Rodent ulcers, 43. Total 132, of which 24 were treated with radon or radium element.

Non-malignant.—Hodgkin's disease, 1; Myelogenous leukæmia, 1; Nævi, 3; Metropathia hæmorrhagica, 3; Tubercular peritonitis, 2; Tubercular glands, 1; Keloids, 2; Exuberant scar tissue, 4; Infected plantar warts, 8; Osteo-arthritis, 3; Various, 4. Total 32. Of these the only cases treated with radon were the two T.B. peritonitis, one nævus, and two various.

No. 24.

STUDIES IN PEAT

Part 11.

PEAT-TAR OILS.

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[Read May 23. Published separately June 15, 1944.]

While the thermal decomposition of peat and bituminous coal would appear to be closely related operations, yet from every consideration practically all advances have been made with the latter material. To take one particular example- the coal-tar distillates from low temperature carbonisation show an extensive literature, while the corresponding peat-tar oils have been examined to a very limited extent.

Some claims have been made for the disinfectant properties of peat-tar fractions by various authors, though experimental evidence to justify these claims is seldom recorded.

Owing to the recent use of peat in gas plants and in charcoal manufacture. with the possible availability of peat-oils, this would appear to be an opportune time for a more extended study of these oils. Except for a preliminary note by Morgan and Scharf (1), very little experimental work appears to have been carried out on such products, especially from Irish peats. In these early reports, no indication is given of the nature of the peat, and there is some evidence that wood and other combustibles were also used in the producer-plant. Part of the lighter oils had also been drained away, and in some cases the samples had undergone changes by long exposure to the atmosphere before examination.

Distillation of Peat.

The peat was air-dried, machine-won peat from Lyrecrumpane, County Kerry. A considerable amount of work has already been carried out at University College, Cork, on this peat in connection with the work on peat waxes (2). Before being placed in the retort the peat was

crushed to pass a sieve of twenty meshes to the inch. The distillation was carried out in an iron retort, gas-heated, and was effected at the comparatively low temperature of 300°–350° C. The condensation system employed was that devised by Donnelly and Reilly (3) to eliminate the disturbing "tar-fog" effect. These authors found that a good fog-trap was provided by a column of tightly packed glass-wool.

Two distillations were carried out, a charge of 1,000 g. of peat being used in each case. When the temperature reached 100° C. it remained constant for about 40 min., water coming over meanwhile also very small quantities of volatile oils which had been steam-distilled. The heating was increased, and the temperature rose rapidly to 300° C. and then slowly to 350° C. The yield of tar-oil distillate from a number of experiments varied from 7 6 to 8 2 per cent. calculated on air-dried peat. Davis (4) mentions that the average yield of tar from peat distilled in retorts is 4 5 per cent. The low temperature carbonisation greatly increases the yield of tar by preventing the cracking of the heavier tar and oils to gaseous matter.

The distillates from the separate experiments were mixed and re-distilled. A further small separation of water was thereby effected. The distillates were extracted with sodium hydroxide solution, and the phenols thus dissolved were re-precipitated with dilute sulphuric acid and then re-distilled to eliminate resinous impurities. For the purposes of examination two fractions were collected, 120°–210° C. and 210°–300° C. The volatile oils of the first fraction were light brown almost yellow in colour, those of the second being of a darker shade. On standing both fractions reverted to an opaque blackish-brown.

The weight of alkali-soluble substances prepared by the above method was 2 14 per cent. of the original air-dried peat. The tar oil was not further refined, since in this form it rapidly gives emulsions. A vacuum distillation of the alkali-soluble fractions was, however, carried out, with results which will be referred to later.

Procedure of Tests.

The term "phenol value" is used in this paper to denote comparative values only. Although the conditions laid down in the standard Rideal-Walker technique were complied with as far as possible, no rigid adherence to this method is claimed. Hence the results here recorded of a series of comparisons against a phenol standard may be regarded as comparative values against phenol of unit value, but only as approximations to the strict bacteriological phenol coefficients.

The test organisms used were 24-hour cultures of *Staphylococcus aureus* and *Bacillus coli,* incubated and grown in nutrient broth at 37° C.

The broth, as used, was prepared by boiling minced cow's heart (from which the fat and fibrous tissue had been previously removed) in distilled water in the proportion of 1 lb. meat to 1,000 ml. water. After 20 min. boiling, the volume was made up to 1,000 ml., filtered, and 10 g. A.R. sodium

chloride and 10 g. pure peptone added. The mixture was again boiled for 20 min., filtered, and adjusted to pH 7·0 to 7 2 with N/10 sodium hydroxide solution. The phenol used for standard comparison had a congealing point of not less than 40° C., and was used in a 5 per cent. solution, being frequently standardised with N/10 bromine. Rimless tubes were employed. To facilitate flaming, lipped Pyrex tubes were used as seeding tubes in some cases. Transfers were made with a 4 mm. (inside diameter) single loop of medium gauge platinum wire. 5 ml. each of the stock dilutions were used per tube. These tubes were placed on the water-bath for 5 min. to bring to the required temperature of 20° C.; 0 5 ml. of the test culture was then added to each of the dilutions at definite time intervals. The culture was added from a graduated pipette holding sufficient material to seed all the tubes in any one set. Unfiltered culture was used, but it was thoroughly shaken 15 min. before use and allowed to settle. To ensure uniformity of conditions during the experiment it too was maintained on the water-bath at a temperature of 20° C.

The medication tubes were agitated, gently but thoroughly, just after the addition of the culture to ensure even distribution. Five minutes after the time of seeding, one loopful of the mixture of culture and diluted liquid was transferred to the appropriate sub-culture tube. At the end of the appropriate time-intervals the same procedure was followed with the second and succeeding medication tubes. Five minutes after the time of making the first transfer a second set of transfers was begun for the 10 min. period, and finally repeated for the 15 min. period. In the later tests, the time intervals were reduced to 2½ min., 5 min., and 7½ min., respectively. The effectiveness of the disinfectant was measured by examination of the sub-culture tubes for signs of growth after incubation for 24–48 hours at 37° C.

Preliminary Tests of Method.

In order to test the comparative results obtained from the technique outlined above and those from the standard test, it was decided to investigate certain substances of known Rideal-Walker coefficient. Tests were carried out against standard phenol solutions with lysol as the test substance. This lysol is stated by Martindale (5) to contain at least 50 per cent. of cresol. According to Hewlett and McIntosh (6) it has a Rideal-Walker coefficient of 2 5, and this figure is claimed for it also by the manufacturers. Details of the media and manipulation employed in this series of tests are here recorded:—

Composition of medium	Broth as described earlier.
"Acidity" of medium	pH 7·2.
Amount of culture medium per tube	10 ml.
Amount of culture added to diluted disinfectant	0·5 ml.
Amount of diluted disinfectant in tube	5 ml.

Condition of tubes in test ...	Plugged with cotton.
Temperature of tubes	20° C.
Time intervals	5, 10 and 15 minutes.
Size of transfer loop	4 mm. diam. (No. 27 Imperial guage).
Calculation of phenol value ...	Highest dilution killing in 10 but not in 5 min., divided by same for phenol.

The average phenol value obtained for lysol by the above method was 1·8.

Preparation of Emulsions.

The oils from the peat-tar distillation had to be emulsified to enable tests to be carried out. After some experiments with oils derived from a mixed coal-peat tar the following was considered the most effective emulsion:—50 parts of phenolic fractions; 20 parts of soft soap; 50 parts of 10 per cent. solution of A.R. sodium carbonate. The mixture was refluxed for 1 hour.

This emulsion passed the standard tests of solubility on dilution:—Two dilutions were prepared: the first containing 1 ml. of disinfectant in 5 ml. of water; the second was that required as a test of purity for higher Liquor Cresolis Saponatus (7) in which 5 ml. of disinfectant was mixed with 95 ml. of water. If no separation appeared in either case after standing for 4 hours, the disinfectant was passed for testing.

Examination for Phenol Value.

Two emulsions were prepared according to the specifications above: the first from the fractions boiling from 120° C.–210° C., and the second from those oils with a boiling point between 210° C.–300° C. For convenience these preparations were labelled P and Q, respectively. The following figures show the calculated phenol values of the emulsions from tests against a culture of *Staphylococcus aureus* resistant to phenol of dilution 1–90 (using time intervals of 5 min., 10 min., and 15 min.):—

Preparation P	Phenol value	9·3
Preparation Q	Phenol value	11·5

The above figures were only very slightly lower than the values obtained when *B. coli* was employed as test organism:—

Preparation P	Phenol value	9·8
Preparation Q	Phenol value	12·8

It appeared that the higher fractions were more potent germicides. A further distillation of the oils was effected, and the small quantity boiling above 250° C. was collected and emulsified as above. This third preparation (labelled R) was tested against *Staphylococcus aureus* to give the phenol value of 14·0.

The peat-tar fractions boiling between 120° C.–300° C. were now placed in a Claisen flask. Glass wool was packed tightly into the flask, which was

evacuated to a pressure of 20 mm. The flask was immersed in an oil-bath which was heated gradually, and the phenols were distilled. The first fractions, which came over in small quantity from 60°–70° C., were colourless oils, which were emulsified and tested as above, using however the time intervals 2½ min., 5 min., and 7½ min. In this case the phenol value was calculated by dividing the highest dilution which shows life in 2½ min. and 5 min., but not thereafter by the same for phenol. This emulsion (preparation S) gave a phenol value of 2·2, against *Staphylococcus aureus.*

Preparations P and Q were also tested under these time intervals, and gave values of 12·2 and 13·6, respectively, also against *S. aureus.*

A blank test was now run on the emulsifying agents used in all the above tests (20 parts soft soap and 50 parts of 10 per cent. solution of A.R. sodium carbonate). Under both time-interval systems the phenol value was found to be in the region of 0 5. (The method of testing used is, perhaps, less accurate with substances of low disinfectant powers).

The following table gives the complete list of phenol values for the various preparations, the figures for the time-intervals 5, 10, 15 min. being in column A and those for 2½, 5, 7½ min. in column B:—

TABLE 1.

	Against *S. aureus.* A	Against *S. aureus.* B	Against *B. Coli.* A
Preparation P	9 3	12 2	9 8
Preparation Q	11 5	13 6	12 8
Preparation R	14 0	14·0	—
Preparation S	—	2·2	—
Emulsifying Mixture	0·5	0·5	—

4-triazo 3, 5-dimethylpyrazole aromatic hydroxyl reactions.

The substance 4-triazo 3, 5-dimethylpyrazole has been shown to develop characteristic and sensitive colour reactions with aromatic hydroxyl compounds (8). Some tests were carried out with this substance on the peat-tar oils used in the preparation of the above preparations with a view to comparing the probable constitution with the apparent phenol values.

Preparation of 4-triazo 3, 5-dimethylpyrazole.

5 g. of dimethylpyrazole prepared from acetylacetone and hydrazine sulphate were heated with 2 ml. of sulphuric acid (d. 1·8) and 4 ml. of fuming nitric acid (d. 1·5), the pyrazole compound being added slowly, and the reaction mixture meanwhile cooled in ice. The solution was then warmed on a water-bath for two hours, and the product poured on to crushed ice. Sodium hydroxide was added until the solution was nearly neutralized, when

4-nitro 3, 5-dimethylpyrazole separated as a thick white precipitate. This was washed with ice-cold water, and then dissolved in 5 ml. of water and 10 ml. of concentrated hydrochloric acid. 5 g. of tin were added gradually, the reaction mixture being immersed in crushed ice. The solution was then heated on a water-bath for two hours, and the tin precipitated as sulphide, after dilution of the mixture. Concentration of the filtrate gave the compound 4-amino 3, 5-dimethylpyrazole in the form of its hydrochloride.

This solution was diazotised with sodium nitrite in the cold (1 2 mols. hydrochloride to 2 mols. nitrite). The excess of nitrite was removed with urea, and 1 5 mols. of sodium azide were added in the cold. After standing for 3 min. sodium carbonate solution was added, and the triazo-pyrazole separated as a bulky white precipitate. It was filtered and washed with cold water, and used without further treatment in the following tests. Small quantities of the peat-tar phenolic fractions were placed in white dishes, and dissolved in dilute alkali. On addition of a small quantity of the triazo reagent to each dish, colorations developed slowly in each case. The following observations were made:—

TABLE 2.

Colour reactions with Triazopyrazole.

Samples.	After 30 min.	After 2 hours.	After 24 hours.
P	Light reddish-purple developed round the edges and slowly enlarged.	Deep purple throughout.	Very deep purple, almost dark blue.
Q	Reddish-violet round edges.	Reddish-purple throughout.	Reddish-purple darkening.
R	Reddish-brown streaks.	Dark blue.	Very deep reddish purple.
S	No coloration.	—	—
Phenol	Bright-blue streaks, purplish at edges.	Deep blue throughout, faintly red at edges.	Very dark-red with bluish tinge.
O-Cresol	Bluish-violet.	Dark red.	Reddish-brown.
M-Cresol	Intense Blue.	Reddish-brown.	Dark brown.

The fractions from the peat-tar oils differ from the lighter hydroxyl constituents of coal-tar, and give mixed colours. In comparative tests the differences were quite distinct, although no quantitative test could be made. It is probable that these peat-tar oils have a much more complex structure embodying a wide range of phenolic substances.

Some Further Tests.

A number of emulsions on similar lines to the above were prepared from oils distilled from a mixed coal-peat tar produced in commercial gas-work carbonisation plants.

A series of twelve tests was performed on these oils, and phenol values ranging from 3·1 to 5·1 were obtained. Further to this, a number of experiments on the partial cracking of these oils were carried out and some interesting results noted.

Cracking of the Oils.

(*a*) *Vapour Phase.*—It was decided to endeavour to effect thermal decomposition without the application of pressure. The first attempts were carried out in the vapour phase. 200 ml. of the coal-peat tar fractions boiling between 0°–250° C. were placed in a distillation flask, the outlet of which was connected to an iron tube, 97 cm. long and 1·8 cm. internal diameter. This tube was filled with unglazed porcelain chippings, and joined to a copper coil immersed in a water jacket. The outlet end of the coil was connected to a pyrex condenser. The pipe was heated to a temperature of 250° C. (measured by means of a thermocouple) throughout its length by a series of gas-jets. The oils in the distillation flask were boiled (208° C.), and the vapour passed through the tube. The distillate was collected in a 20 per cent. solution of sodium hydroxide, the alkali-soluble fractions being later salted out. In all, about 170 ml. of oils passed through the pipe and yielded 98 ml. of alkali-soluble distillate. The boiling point of the distillate was 187°–189° C.

Two separate disinfectant preparations were now made up according to the specifications already given for the uncontaminated peat-tar oils. The first, prepared from the uncontaminated coal-peat tar phenols, was called Preparation A, and the second, prepared from the same oils after they had undergone thermal decomposition, was called Preparation A_1. The phenol values of these emulsions were now investigated against staphylococcus and over 5, 10, and 15 minute intervals, and the following figures were obtained from a series of four complete experiments. It will be noted that the phenol values were not constant throughout, nor did they show a constant increase from A to A_1, owing to the varying amounts of coal-tar in the different original tar samples, but in all cases the figures for A_1 were higher than those for A.

Experiment No.	Phenol value of A.	Phenol value of A_1.
1	4·3	7 4
2	6 2	11 8
3	5 2	7 0
4	5·1	7·2

(*b*) *Liquid Phase.*—It was now endeavoured to affect a similar molecular rearrangement in the liquid phase. In this instance the oils were dropped slowly from a funnel on to an iron plate heated from 350°–380° C., and the vapour condensed and collected in alkaline solution, from which the phenols were extracted as before. This process gave a 77 per cent. yield of alkali-soluble tar oils which boiled at 204° C. A preparation of these oils (emulsified in the

same way as before) was labelled A_2. Various samples so prepared were placed in comparison with corresponding samples of A and A_1 (against *Staphylococcus aureus* and over 5, 10, 15 minute intervals). The results are tabulated below.

Test No.	Phenol value of A.	Phenol value of A_1.	Phenol value of A_2.
1	5·6	7·2	5·4
2	4·8	6·8	5·2
3	4·8	6·4	5·4

Similar experiments to the above conducted with uncontaminated peat-tar oils did not provide similar results, but further work is in progress on this aspect of the problem.

Summary.

Peat on distillation at 300°–350° C. yielded approximately 8 per cent. of peat-tar. The tar was fractionated, and the various fractions extracted with alkali. The extracts emulsified readily, and in preliminary tests of approximate bactericidal power gave high values when compared with phenol and lysol preparations. From qualitative colour tests with 4-triazo 3, 5-dimethylpyrazole, there is indication that substances other than simple phenols or cresols are present. Further work is in progress in the preparation of emulsions of higher tar-oil contents.

Cracking experiments to increase the activity of the oils give positive values with the mixed coal-peat tar fractions, but so far this treatment has not improved the peat-tar oils.

These preliminary experiments suggest the need for further experiments on the bacteriological examination of peat-tar oils prepared under different conditions.

Acknowledgments are made (i) to Mr. V. J. Albericci. M.SC., for the emulsions of mixed coal-peat tar oils; (ii) to the Pathology and Dairy Science Departments, U.C.C., for supplying the test cultures; and (iii) to the Irish Industrial Research Council for a grant to one of us (D.R.).

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No. 25.

STUDIES IN PEAT.

PART 12.

MONA WAX (IRISH PEAT WAX) AND EMULSIFICATION.

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(PLATE 10.)

[Read MAY 23. Published separately JUNE 15, 1944.]

IRISH peat containing as much as 45 per cent. of water can be extracted with petroleum-hydrocarbon solvents. Reilly and Emlyn (1) accounted for the extraction on the assumption that water-in-oil (W/O) emulsions are formed in the initial stages of the extraction, the water being consequently unable to prevent intimate contact of the solvent and the peat-wax. Finely divided carbonaceous matter in the peat was given as a possible emulsifying agent. It was also stated (*loc. cit.*) that very stable W/O emulsions could be formed with the molten wax as the continuous phase.

It has now been found that the wax extracted from peat by any of the solvents (e.g. petrol-alcohol, trichlorethylene, etc.) used in its production is capable, when present in the "oil" phase in relatively small amounts, of permitting water to be dispersed in certain hydrocarbon liquids (e.g. benzene, toluene, liquid paraffin). When the percentage of water dispersed in the oil is high (e.g. 75 per cent. of the total volume), the product has a consistency approaching that of a paste.

During the course of the latest experiments it was found that the products resulting from the dispersal of water in a wax-containing oil were always of yellow-brown colour, and they appeared to contain a voluminous precipitate. Examination under the microscope, however, showed them to be true emulsions. On being tested for type, these emulsions were found to be uniformly of the W/O variety. On standing in closed vessels, for periods up to eight weeks, these emulsions proved to be fairly permanent in the sense that no gross deterioration appeared to occur.

The peat-extract as a whole has, in previous papers, been designated "Mona wax," and this title will be used in subsequent description and reference.

The carbonaceous matter, originally contained in the peat, may have been mechanically collected in the extract, and may, perhaps, be suggested as the constituent which is effectual in the production of the wax-sponsored emulsions. Since, however, the percentage of this carbonaceous matter in the wax would, at most, be small, it is unlikely that the quantity of such carbonaceous material present, for example, in 0 075 gram of Mona wax, would sponsor an emulsion consisting of 60 ml. of distilled water dispersed in 20 ml. of an oil. It is possible, however, that during the initial stages of the extraction of moist peat by petroleum-hydrocarbon solvents, traces of carbonaceous matter in the ground peat may be responsible for the emulsification in the oil of "peat-moisture," thus allowing more intimate contact of the solvent and the wax-material. If this phenomenon were to occur even "locally" within the mass of the ground peat, and were a small quantity of the wax, as a result, to be extracted by the solvent, this extracted wax would assist in the emulsification of some of the remaining water. Such a process would be in keeping with the fact that, under certain conditions, the wax is capable of maintaining fairly concentrated water-in-hydrocarbon emulsions of low wax/oil ratio. The removal of this moisture would, in turn, aid in the extraction of more of the wax which, accordingly, would assist in the emulsification of more of the water. The effect, therefore, would be cumulative.

Since traces of carbonaceous material cannot be held to be the only possible constituent of Mona wax responsible for the maintenance of concentrated W/O emulsions, other factors have to be considered. From a study of the literature it would appear that no systematic investigation of the emulsions sponsored by peat-wax has been attempted. It was therefore necessary to prepare such systems, and to make a study of some of their characteristics.

Emulsions of water in hydrocarbon-oils were prepared, using Mona wax as the emulsifying agent. The hydrocarbon-oils used were benzene, toluene, xylene, and liquid paraffin. On account of its low volatility, liquid paraffin was the oil most commonly used in the work undertaken. It was found possible to prepare emulsions containing as much as 79 per cent. of dispersed water. Downward creaming[1] was a feature of the emulsions of water-concentration of even 75 per cent. With such a high water-content, however, the separated oil-layer occupied but a small bulk. That creaming should have taken place at all in such a concentrated emulsion is accounted for by the irregularity of the water-globules both in size and (often) in shape. An emulsion of 79 per cent. water in 21 per cent. liquid paraffin, with Mona wax as emulsifying agent, showed no evidence of creaming on being set aside for five weeks. Creamed emulsions were readily remixed by inverting the container a few times, allowing the stiff cream to run (when necessary), followed by gentle shaking of the container.

[1] The term "creaming" is used in the usual sense, viz., the separation, due to the influence of gravity, of a more concentrated emulsion from a dilute emulsion, i.e. the formation of two distinct layers, one consisting of the more concentrated emulsion, the other being the impoverished dilute emulsion (or even the clear external phase).

The emulsions, which had widely varying concentrations, were prepared by the intermittent-addition intermittent-shaking method (see later). It was observed on a number of occasions that when concentrated emulsions (75 per cent. water with liquid paraffin as the continuous medium and Mona wax as the "stabiliser") were allowed to stand for one hour, and then submitted to about thirty rapid continuous shakes, partial breaking of the emulsions resulted, bulk-quantities of water, estimated at from 5 per cent. to 20 per cent. of the total water in the system, being visible within the container. On setting these partially-broken emulsions aside for a short period, and then reshaking, most of the separated water disappeared.

Attempts made to homogenise water-in-liquid-paraffin emulsions by passing them through a hand-operated cream-maker resulted in partial breaking and partial homogenisation. On gently rubbing small samples of the concentrated emulsions the breaking of the emulsion was evident from the visible droplets of water which collected at intervals throughout the extended surface. More dilute water-in-liquid-paraffin emulsions (e.g. 40 per cent. water) failed to break when submitted to rapid continuous shaking, but the gentle rubbing of small samples again caused the appearance of small droplets of water. Emulsions of water in benzene and in toluene with the same emulsifying agent failed to break, irrespective of concentration, when subjected to rapid continuous agitation.

Until recently, the term "stability," as applied to emulsion-systems, lacked a sufficiently rigid significance. In 1939 King and Mukherjee (2), who then worked with soap-stabilised emulsions, defined the "stability factor" of an O/W system as the reciprocal of the rate of change (with respect to time) of interfacial area, per unit area of initial emulsion interface. In this connection the term "specific surface" ("specific interface," "specific interfacial area") may be expressed as the number of square decimetres of interfacial area per gram of dispersed oil. The authors assumed that the rate of change of the specific surface of an O/W emulsion is proportional to the initial specific surface (although in the subsequent work (3) they found, in some cases, that there appeared to be two rates—an initial rapid change, followed by a slower change), i.e., according to the simpler assumption,

$$-\frac{ds}{dt} = k_1 s_1 = \frac{s_1}{k},$$

where s = specific surface; s_1 = initial specific surface; t = time; k_1 = instability factor; k = stability factor.

They developed a method for the evaluation of k, based on size-frequency analyses, and were also able to compare the efficiencies of certain emulsifying agents when the concentrations of the agents were not too small. The work was later extended (3) to cases of emulsions of the O/W type stabilised by other hydrophilic colloids. As part of the analysis technique a representative sample of each given emulsion was suitably diluted, and the diluted sample

(where a soap was the emulsifying agent) was mixed with a little aqueous gelatine solution. One drop of the mixture was then placed on a microscope-slide, protected by the cover-glass, and the gel allowed to set (the gel was formed for the purpose of removing disturbances due to Brownian movement and to drift caused by convection, etc.).

The magnified image of the sample, thus treated, was projected on to a rigid screen of squared paper. This process enabled globule diameters to be measured. By choosing a statistically sufficient number of fields from a given sample, the total number of globules could be counted and arranged into size-groups.

Assuming that there were n_1 globules of mean diameter d_1, n_2 globules of mean diameter d_2, etc., the relative total volume, V, for all size-groups would be given by the expression $\Sigma n.\frac{\pi}{6}.d^3$, and the relative total surface area, A, by $\Sigma n.\pi.d^2$. The specific surface would then be given by $A/\rho V$ where ρ is the density of the dispersed liquid.

By such size-frequency analyses on a given batch of emulsion, after various suitable time-intervals, the value of the stability factor was calculated.

Lotzkar and Maclay (4) determined the comparative efficiencies of certain emulsfying agents by comparing the stabilities of O/W emulsions sponsored by these agents. The technique used by these workers involved the use of a hæmacytometer-slide. Specific surfaces were measured, from photomicrographs, in the same manner as in the previous case, but the formula used in calculating the stability-factor (k) was modified. These authors used the equation:

$$-\frac{ds}{dt} = k_1 s = \frac{s}{k}.$$

It was decided, in the present work, to investigate the "stabilities" of emulsion-systems in which liquid paraffin was the external phase, distilled water the dispersed liquid, and Mona wax the emulsifying agent. While it was appreciated that certain approximations would have to be made in the determination of globule-diameters (owing to the somewhat irregular shapes of some of the globules), an analysis on the lines already indicated was attempted.

A number of more dilute emulsions of water in liquid paraffin were prepared. One such emulsion, prepared by the intermittent-addition intermittent-shaking method, followed by a number of continuous shakes, consisted of 16 ml. of distilled water dispersed in 24 ml. of liquid paraffin in which 0·2 g. of Mona wax (extracted from peat by trichlorethylene) had already been dissolved. The emulsions thus prepared were fairly viscous. They were usually allowed to stand for one hour before being examined under the microscope. A drop of such an emulsion, examined under the low power without the use of a cover-glass, showed that the dispersion was somewhat

coarse. Fig. 1 is a photomicrograph of a representative field selected under the low power, of a sample of the emulsion to which particular reference has just been made, the sample being placed on a flat slide, and no cover-glass being used in the process. Fig. 2 is a photomicrograph showing the effect produced on the careful addition of a light cover-glass. It will be observed that some deterioration of the emulsion-sample has occurred. For more critical examination the emulsion was diluted with the continuous phase (liquid paraffin). A one-in-four dilution was found suitable for low-power work, while a one-in-eight dilution was suitable for high-power scrutiny. Under the high-power it was observed, in the cases of globules large enough to make such observation possible and when the upper surfaces of such globules were correctly in focus, that the globules were surrounded by films which had a cracked or wrinkled appearance (5). The films resembled "cellophane wrappings." Fig 3 is a photomicrograph showing some of the larger globules with the films in focus. No cover-glass was used in the high-power work just described. Even the most cautious subsequent addition of the cover-glass resulted in the breaking of these films and the coalescence of the water-globules in "pools." Minute scrutiny of the complete field revealed the presence of but few surviving globules. Samples of more viscous concentrated emulsions were tested for the effect of cover-glass addition. Breaking of the films also occurred in these cases, but to a minor degree. For certain other observations, use was made of a microscope-slide with round cavities.

Samples of emulsions of water in liquid paraffin, when examined immediately after preparation (i.e. without a rest-period) were found to contain globules nearly all of which were spherical in shape. While an emulsion under examination was still fresh, no cracked or wrinkled interfacial films could be observed. The films usually began to make an appearance when the emulsion had been resting for about 40 minutes.

A 40 per cent. emulsion of water in liquid paraffin, with Mona wax as the emulsifying agent, was prepared and was examined in the container, by unaided vision, at regular intervals, over periods of weeks. Apart from creaming, which was relatively rapid (as with most W/O emulsions), no gross change in the system could be observed. Samples of the same emulsion were subjected to microscopic examination at intervals over the same period. For this purpose the creamed material was gently re-mixed, representative samples withdrawn, and the samples placed gently on a slide. When a flat slide was used no cover-glass was added. When a slide with round cavities was employed, the sample added was insufficient to fill the cavity completely; a cover-glass could then be used. As a result of this microscopic examination no evident gross deterioration of the emulsion, over the period of examination, could be observed. Similar experiments with a duplicate batch of separately-prepared emulsion yielded similar results.

The breaking of these emulsions due to the addition, to samples on a flat slide, of a cover-glass, is further illustrated in the accompanying Figures (4

and 5). Fig. 4 is a photomicrograph of a representative field, taken under the high-power, of a diluted sample (1 in 8) of a 40 per cent. emulsion, aged 17 days, no cover-glass being used. Fig. 5 shows the deterioration which occurred on the careful addition of a cover-glass to the same sample.

At this stage of the work it became questionable as to whether the terms "stable" and "stability" could, even in the ordinary restricted sense, be applied to such a system. In cases where the dilute emulsion was set aside and subjected to no greater mechanical disturbance than the reasonably gentle re-mixing of creamed material, it was certainly established both by unaided vision and by microscopic examination that the system (apart from the inevitable creaming) showed good permanence for periods of weeks. The effect of the cover-glass addition, however, proves that the films which enveloped the globules could have had very little mechanical "strength" (6).

Dilution with liquid paraffin might have exerted some solvent action on the films which had already formed around the water-globules, thereby causing a certain amount of film-weakness. This was tested by diluting the emulsions with liquid paraffin containing the same percentage of dissolved Mona wax as that originally used in the preparation of the emulsion. No noticeable change in film-strength was observed as a result of this change in the nature of the diluent. These preliminary experiments in connection with the size-frequency analysis give some idea, independent of the question of "stability," of the nature of the films which enveloped the water-globules.

By taking, on a flat glass slide, a sample of a water-in-liquid-paraffin emulsion which contained about 40 per cent. of dispersed water, and adding the cover-glass, it was found possible, in some cases, by gentle pressure applied to the cover-glass by means of a needle, to obtain a field which contained globules large enough for the observation of some of the characteristics of the film. In some cases these globules seemed to be attached, through the agency of the film itself, to one of the glass surfaces. In other cases the globules were free to move. It was observed that, on touching the cover-glass, the film tended to change its shape and the enclosed water appeared to move, within the film, from one position to another. It was concluded that the film was somewhat sticky, and that the marks on the surface were wrinkles and not cracks. The adhesive characteristics of the film were further evidenced by the tendency, noticed in a number of instances, of smaller globules, already attached to larger ones, to remain thus attached.

During one particular examination of a water-in-liquid-paraffin system the possibility of the occurrence, in such systems, of emulsions of the "multiple" kind was demonstrated. One large film-covered globule contained, within itself, a number of apparently uniform smaller globules. Near the upper inner surface about five of these smaller globules could be counted, but on focussing below this level the smaller globules appeared to be very numerous and closely packed. Assuming that the large globule contained water as the "external" liquid, it could not be decided whether the smaller globules consisted of liquid paraffin surrounded by protective films (thus preventing

their coalescence) or of water surrounded by films (thus preventing inter-coalescence and mixing with the continuous water within the large membrane).

The process of film-rupture has been recorded by photomicrographs. Fig. 6 shows a globule in which the film has just broken at one side. Water (in focus) can be seen issuing through the broken part of the film. Fig. 7 shows a number of collapsed films (without water).

With regard to the permanence of the concentrated water-in-liquid-paraffin emulsion systems, it is likely that when the film is not subjected to undue mechanical stress it is sufficiently tenacious to prevent rapid coalescence of the water globules. In these cases the high viscosity of the system helps to prevent a too rapid impact of the globules when relatively gentle methods of re-mixing are employed. The rapid continuous shaking (involving violent contact with the inner surface of the container) was, however, vigorous enough to cause a serious amount of film rupture. It may be argued that it is a matter of hazard whether a given type and extent of shaking will, during the process of the making of the emulsion, succeed in emulsifying or de-emulsifying the given mixture. However, in no case did the intermittent-addition intermittent-shaking method used give any difficulty in the matter of the preparation of emulsions up to fairly high concentration limits (7), and in no case was the phenomenon of bulk phase-inversion experienced during the course of the preparation of the emulsions by this method. It may again be mentioned that interfacial films did not actually appear for some time after the emulsions had been prepared.

Whilst the above explanation of the permanence of concentrated water-in-liquid-paraffin emulsions seems reasonable, it must be admitted that no breaking occurred when more dilute emulsions of the same constituents were submitted to violent continuous shaking It is possible that when the concentration of the water in the system is low, the film absorbed may be stronger. When benzene or toluene constituted the external liquid phase, violent continuous shaking of the system (dilute or concentrated) also failed to break the emulsion. Preliminary investigation of water-in-toluene emulsions indicated that the mechanical stability of such systems may be enhanced through an additional cause These systems are receiving further attention by hanging-drop slide technique, etc. The effectiveness of various constituents of Mona waxes, and of treated Mona waxes, in emulsification processes, and of the effect of the presence, in the water, of selected electrolytes, are also being investigated.

Preparation of the emulsions.—Emulsions were usually prepared in 100-ml. Pyrex test tubes which were fitted with corks. The desired volume of oil was measured into the test tube, and a weighed quantity of the wax added. The wax was then dissolved by warming and gentle shaking. In some cases the inner walls of the tube were wetted with the solution thus made, but it was found that, in general, such a precaution was not essential for success in the preparation of these W/O emulsions. The solution was then brought to an arbitrary temperature (40° C. when liquid paraffin was used) and maintained at or near this temperature during the preparation of the emulsion. The

addition of the distilled water was carried out intermittently, the shaking being also intermittent, as described below.

Addition and shaking.—For the emulsification of 16 ml. of distilled water in 24 ml. of liquid paraffin (ρ = 0·894/15 5° C., η = 252 Redwood No. 1 seconds at the same temperature) containing Mona wax:—2 ml. of distilled water, at approximately 40° C., was added to the liquid paraffin solution of the wax, the tube corked, and the system submitted to three sets, each of three vigorous up-and-down shakes. After a short pause another 2 ml. of the warm distilled water was added, and the system submitted to similar shaking. A third 2 ml. was then added and the shaking process was repeated. The remainder of the water was then added in two 5-ml. portions, the tube and contents receiving the same shaking treatment after each 5-ml. addition as was received after each 2-ml. addition. After the final shake of this series the system was allowed to rest for 1 minute, and then given six additional up-and-down shakes, individually vigorous, but with a slight pause between successive shakes. The emulsion thus formed was then set aside to cool in the corked test tubes. In most cases a period of at least one hour of standing had elapsed before submitting the emulsion to tests.

For the emulsification of 60 ml. of water in 20 ml of the liquid paraffin containing Mona wax:—A procedure similar to that indicated above was followed, the addition of the distilled water being made in five 2-ml. quantities followed by ten 5-ml. quantities.

Weights of wax used.—Experiments were performed using various weights of wax per given volume of liquid paraffin. Thus, for 20-ml. volumes, the following weights were used:—0·075 g., 0 1 g., 0·125 g., 0·15 g., 0·175 g., 0 2 g., 0·25 g., 0 3 g., 0·35 g. Qualitatively, all these weights of wax gave fairly good emulsions, but the best emulsion resulted from the use of 0·2 g. per 20 ml. of the oil. It was therefore decided to use this proportion (or an approximation to it, e.g. 0·2 g. in 24 ml.) in the more extended work.

Determination of Type.—Various methods were used, all of them being sometimes applied to samples of the same emulsion, e.g. the Dye Method (slowly spreading colour with specks of Sudan III), the Dilution Method (dilution with the liquid which forms the external phase), Clayton's Electrical Conductance Method (a blank being performed on the water used), Direction of the Creaming (used in the case of the more dilute emulsions).

Homogenisation.—A small, hand-operated cream-maker was employed. The apparatus was fitted into a rectangular water bath, the water of which could be heated electrically to any desired temperature. The "cream" outlet passed through the base of the bath.

Photomicrography.—Photomicrographs of representative fields were taken by means of a simplified apparatus consisting of a microscope and a camera. The microscope was fitted with a Leitz × 12 Periplan ocular. A 1/6″ Watson Panchromatic objective was employed for high-power work, and a 1″ Swift objective for low-power work. The microscope-tube was extended to 155 mm. A normal miniature camera with a 5-cm. lens, operating on 35-mm. perforated

film, was used, the lens being set at the infinity mark. In addition to the 50-mm. extension due to the camera, there was an extension of 16 mm. due to a lens-hood attached to the camera. Slides were examined in a horizontal plane to minimise movement of globules, and when a suitable field had been chosen, the camera was clamped rigidly in position and the exposure made. The degree of linear magnification obtained on the film was 110 when the high-power objective was used, and 15·4 when the low-power objective was employed.

SUMMARY.

Mona wax acts as an emulsifying agent which sponsors the emulsification of water in hydrocarbon oil. Emulsions of water in liquid paraffin containing up to 79 per cent. of water have been prepared and examined. The mechanism by which moist peat is readily extracted by petroleum-hydrocarbon solvents has been accounted for in terms of the effectiveness with which the wax itself acts as an emulsifying agent.

The intermittent-addition intermittent-shaking method resulted in the satisfactory emulsification of water in the oils chosen.

The existence, in the case of emulsions of water in liquid paraffin (where Mona wax was used as emulsifying agent), of plastic-like, interfacial films, surrounding the water-globules, has been demonstrated. Such films have but little mechanical strength. A suggestion as to the mechanism of the breaking, by specified methods, of concentrated water-in-liquid-paraffin emulsions (sponsored by the wax), has been put forward.

While the emulsions were reasonably permanent, in the sense outlined, it is considered that any coefficient arrived at by the methods of size-frequency analysis (due precaution being taken against submission of the interfacial membrane to undue external mechanical disturbance) might, in the case of such systems, be termed a "factor of permanence" rather than a "stability factor."

DESCRIPTION OF PLATE 10.

FIG. 1.—Water-in-liquid-paraffin emulsion—Mona wax as emulsifying agent. Without cover-glass; × 42.

FIG. 2.—Effect produced on gentle addition of cover-glass to sample; × 42.

FIG. 3.—Interfacial films on emulsion globules; × 300.

FIG. 4.—Diluted sample of water-in-liquid-paraffin emulsion (aged 17 days); Without cover-glass; × 300.

FIG. 5.—Effect of adding cover-glass to diluted sample; × 300.

FIG. 6.—Process of interfacial-film rupture. Issuing water in focus; × 300.

FIG. 7.—Collapsed films, without water; × 300.

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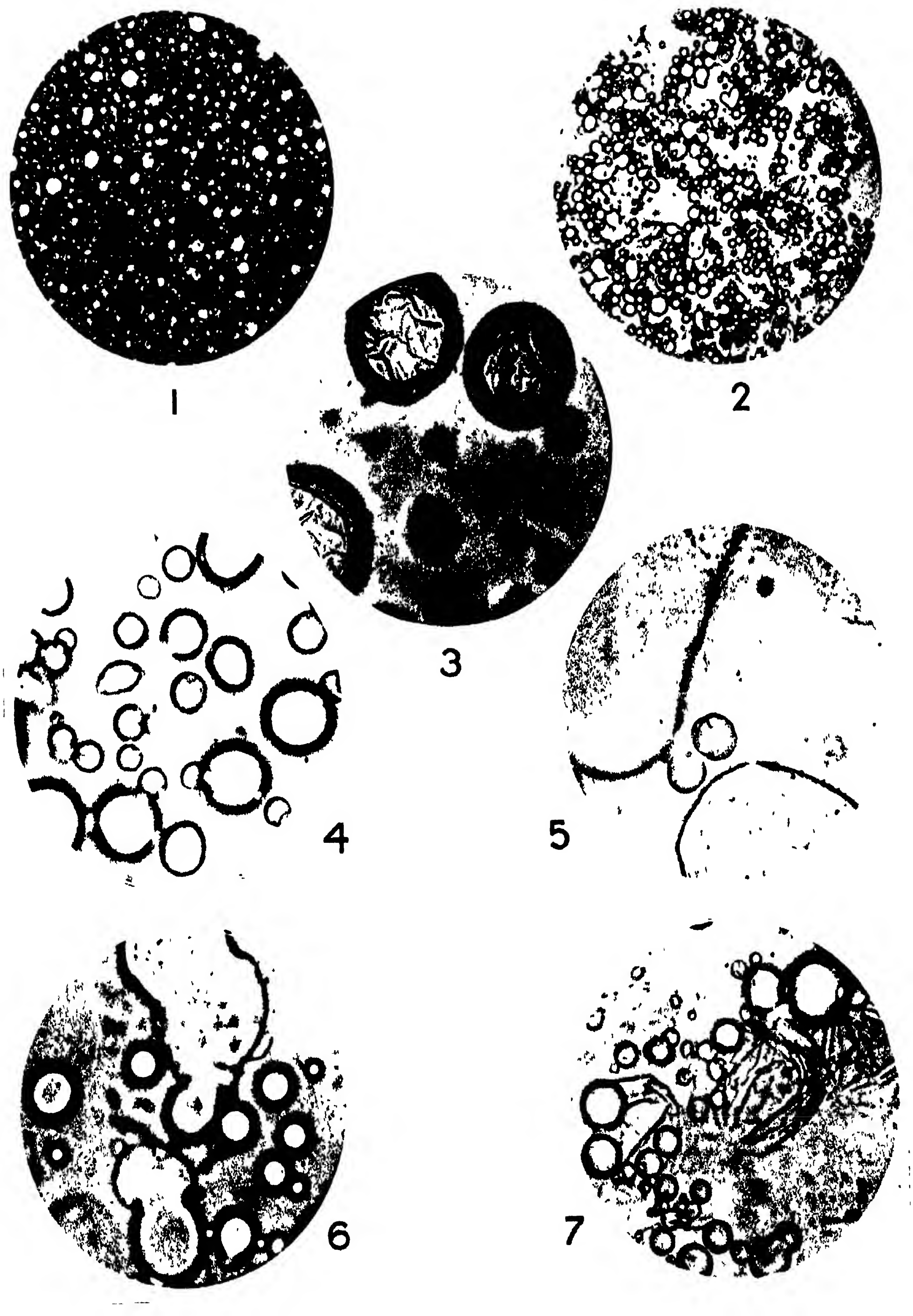
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No. 26.

FERTILIZATION AND EARLY EMBRYOGENY IN *PODOCARPUS ANDINUS.*

By W. J. LOOBY and J. DOYLE.

(Plates 11 to 14.)

[Read June 27. Published separately July 27, 1944.]

This paper continues one recently published (Looby and Doyle, 1944) in which an account was given of the gametophytes of *Podocarpus andinus* Poeppig up to fertilization. In that paper the interest of the relation of this species to other podocarps, the pertinent literature, the source of the material, and the like were described. Here the development is further followed to include fertilization, proembryo, and early embryogeny.

(A) *Fertilization.*—The structure of the mature archegonium and the nature of the two very unequal male cells are fully described in the earlier paper.

At maturity a discharge of cytoplasm takes place between the neck cells (Fig. 1), drawing the female nucleus up to a point, and forcing the male cells back to the top of the canal made by the downward passage of the pollen tube. The discharge area is shown more clearly in Fig. 2. Immediately above the dark discharged mass lies a layer of pollen tube cytoplasm in which a number of the sterile nuclei are still visible. This phenomenon seems unique in conifers. Owing to the definite granulate structure of the archegonial cytoplasm the remnants of the discharged material can be readily recognized in later stages. It is thus normal and not due to unsuitable pressure on turgid archegonia during the collecting.

The next stage available, Fig. 3, showed the female nucleus contracted back and the male nucleus, indicated by its dense nucleolus, passing between the neck cells. The main mass of the discharged cytoplasm in this case was to be seen in a nearby section of the ribbon. The functional male nucleus, with some at least of the male cytoplasm, comes to lie near the large archegonial nucleus, Fig. 4, and soon becomes partly embedded in it, Fig. 5. The large dark body in this upper male nucleus is its characteristic nucleolus. Fig. 61 shows the relations of these nuclei to the whole archegonium. The male nucleus coalesces with the female one, Fig. 6, the nucleolus disintegrating and the delimitation between the nuclei disappearing, thus leading to the typical fusion nucleus, Fig. 7. Fusion of two such nuclei as these offers a difficult cytological problem. Many other stages were available, but as the essential nature of the phenomenon cannot be explained these stages are omitted. The male cytoplasm which entered appears as the darker zone to the right of the male nucleus

in Fig. 4, above the fusing nuclei in Figs. 5 and 6, and to the left of the fusion nucleus in Fig. 7, but it never forms a special sheath. The second smaller male nucleus may be left outside, but it commonly enters; the small dark body above the fusing nuclei in Figs. 5 and 6 is its nucleolus. The sterile nuclei from the pollen tube also commonly enter, but they are ephemeral and their identification usually uncertain as nuclei from the jacket cells make their way into the top of the archegonium about this stage. They are shown in Fig. 8, the clump of tiny nuclei just above the large fusion nucleus of which only part is included.

The only reference to development in *Stachycarpus* is the short incomplete survey by Sinnott (1913) of some stages in *P. spicatus* and *P. ferrugineus*, but no data on fertilization are given. A drawing is given, for the second of these species, of a stage, said to be just before fertilization, which shows a pollen tube with male cells, over an archegonium. Only a small nucleolus is drawn in the functioning male cell, and it is thus uncertain whether this male cell is still undeveloped or whether the enormous nucleolus of *P. andinus* is peculiar to this species. He also states that the male nucleus is about one half the size of the female nucleus. Actually, in his drawing the diameter of the female nucleus is roughly four times that of the male, the former being thus about sixty-four times larger than the latter. The marked inequality in the size of these nuclei in *P. andinus* is evident from Figs. 4 and 5.

(B) *The proembryo.*—The first division of the fusion nucleus, which has been figured by Sinnott (1913) for *P. spicatus*, takes place in the upper part of the archegonium. The mitotic figure, minute and intranuclear, is shown with reference to the whole archegonium in Fig. 9, and in relation to the fusion nucleus in particular in Fig. 10. In both it is indicated by an arrow, the dark body above it being a protein body displaced from the cytoplasm in cutting. The problem of the relation of chromosome to resting nucleus is certainly presented here in an acute form! The two nuclei so formed slip out from the remains of the fusion nucleus, still visible at the top of Fig. 11, and pass some way to the middle of the archegonium, Fig. 11, in which position the second division takes place. The location of this division in relation to the whole archegonium is shown in Fig. 62, the divisions, simultaneous and again intranuclear, in Fig. 12. In this case the nuclear substance surrounding the mitoses rapidly disappears as such, and, even while still in metaphase, the mitoses may appear to lie merely in a slightly altered zone of cytoplasm (Fig. 13). Three of the resultant nuclei shortly after completion of telophase can be seen in Fig. 14 lying in a special zone of cytoplasm, the residue of the fusion nucleus being still visible above. The four nuclei, with a certain amount of cytoplasm surrounding them, continue to move towards the archegonial base and increase in size (Fig. 15). In the meantime the basal third, or less, of the archegonium becomes more compact than the upper part, which is loosely vacuolate and shows increasingly an appearance of marked senescence, somewhat as is figured in a later stage in Fig. 64. When the

proembryo nuclei reach the top of the denser basal zone they lie clumped together (Fig. 63), but soon slip free from any surrounding cytoplasm and pass cleanly into the basal area. Two of the nuclei are shown well down in this zone in Fig. 16. A further carving out of a still denser area of cytoplasm smaller than the first now takes place, as seen in Figs. 17 and 18. In the section from which Fig. 17 was taken this zone is cut a little tangentially but shows three of the nuclei, Fig. 18 showing the form of this final embryonic area better, although the nuclei, being in a deeper focus, are only indicated. In this small area the subsequent stages in the proembryo take place, and during most of this period, owing to the denseness of the cytoplasm and its great avidity for any dye, differential staining proved difficult. The size of this area relative to the whole archegonium is shown by the small black tip of the right-hand archegonium in Fig. 64.

Sinnott (1913) mentions the passage of four proembryo nuclei to the base of the archegonium in *P. spicatus* and *P. ferrugineus*, but his account of the subsequent stages is so brief and inadequate that no further reference need be made to it.

Three further successive simultaneous divisions now take place, resulting in 32 free nuclei before wall formation. The series of figures which follows to illustrate this is a selection from a much more complete series of stages, and is used to show that the development was examined fairly completely. The four mitoses of the third embryonic division are shown in Figs. 19 and 20. Figs. 21 to 23 are from one proembryo at the 8-nucleate stage, five nuclei being shown clearly and the other three more faintly, the remaining sections from this proembryo being omitted. Fig. 24, the same stage as Fig. 22, but at a lower magnification, shows the relations to the lower part of the archegonium. The mitoses of the fourth division are given in Figs. 25 and 26, parts of two mitoses appearing in each. Figs. 27 and 28 show two sections from another proembryo at this stage, but at late telophase, the small black dots being the chromosome clusters. Nine such clusters can clearly be seen, the other seven being equally clear in sections here omitted. The sixteen nuclei so formed enlarge, but remain scattered without obvious order, and show no sign of walls or cleavage between them. Fig. 29, in which nine nuclei can be counted, is from one section of a proembryo at this 16-nucleate stage. The fifth simultaneous division itself was, unfortunately, not found, but a stage very shortly after it was obtained. It might have been advisable to present a more complete series here, but, as a long series would be necessary to demonstrate the full number of nuclei and their organization, only a limited number of illustrations are included. One section from the youngest 32-nucleate stage available is shown in Fig. 30. Here the nuclei are small and obviously only recently formed, while some still tend to lie in pairs. These nuclei as they enlarge become organized with some regularity, but all the nuclei are not included in this organization. Fig. 31 is a rather tangentia[l] section of another slightly older stage in which the nuclei are already coming to lie in some order, and, above the functioning upper tier, at least four nucle[i]

can be seen lying loosely in the cytoplasm. These may be referred to as the *relict* nuclei. The number of the lower compacted nuclei is always less than 32, perhaps as low as 22. While it is not easy to count accurately a large number of nuclei in dense cytoplasm with poor differential staining, yet the total counts in the preparations available varied from 30 to 33 if the relict nuclei are included. These relict nuclei tend to disintegrate rapidly. Two can be seen above in Fig. 30, one of which is already large and faint while the other shows a premature mitosis. This latter appearance was frequently seen, but the mitosis is seldom, probably never, completed, the chromosomes breaking up to irregular dark masses. The number of relict nuclei varies roughly from 6 to 10, and thus the number of nuclei taking part in the proembryo proper must vary from about 22 to 26 or so.

The functioning nuclei become arranged in tiers, of which only the upper is commonly well defined. Figs. 32 and 33 (on the left; the group on the right is an 8-nucleate stage in the other archegonium) show stages in the arranging of the nuclei shortly before cleavage. It is clear from Fig 33 that the tip is occupied by a single nucleus. No membranes are formed on the spindles of the last division, and wall formation seems to be by cleavage planes. Material did not suffice to follow the cytology of this cleavage, which is sufficiently indicated for present purposes in Fig. 34. The form of the young proembryo, as shown in Figs. 35 and 38, is rapidly reached. The upper tier of nuclei have formed a group of elongating cells still open above, the others forming an embryo group of completely walled cells, a large terminal cell being very conspicuous. Differential staining is still uncertain, but the large nuclei of the upper tier and the nucleus of the cap cell can be seen in these two figures, while Fig. 36, another section from the same proembryo as Fig. 38, suffices to show that all the cells are still uninucleate. This section was obviously one tangential to the embryo, and the figure includes only embryo cells at a higher magnification, the edge of the cap cell being just indicated below.

The nuclei of the upper open tier divide more or less simultaneously to form the prosuspensor tier proper, and another tier of cells above open to the archegonium. This tier could be called the *open-cell* tier. An early stage in its formation is shown in Fig. 39, and one of the many later stages available in Fig. 41. This tier is not permanent, and may be quite ephemeral. At the same time, or perhaps a little later, the nuclei of the embryo group, with the probable exception of the terminal cell, divide, again more or less simultaneously, to give rise to the characteristic podocarpean feature—the binucleate condition of these proembryo cells. Fig. 40 shows these cells from Fig. 39 at a higher magnification. The focus is on the two upper cells, in which the mitotic figures are clear, but in all the cells when focussed successively some stage in division can be seen. The resulting binucleate condition of the cells is shown in many of the succeeding figures, notably 42, 43, and 51. In only one case were two nuclei definitely determined in the terminal cell. This condition may have been obscured in some cases owing to the denseness of the cytoplasm, but certainly in many cases only one nucleus was present. The nuclei in the

embryo are at first difficult to separate from the cytoplasm, but when this can be done they show the simple rather undifferentiated appearance recorded in Fig. 42, which should be compared with the very different nuclei of Fig. 43, a difference which will be explained shortly.

As already mentioned the number of nuclei and resulting cells in the proembryo may vary, and of these the numbers forming the embryo group and prosuspensor tier, respectively, may again vary within wide limits. Judging from the examination of about 36 proembryos before suspensor elongation the number in the upper tier may vary in general between 9 and 13, once being as low as 6 and once as high as 15. In most cases the number in the embryo group lies between 11 and 15, with an occasional 9 or 10 and once dropping as low as 7. The proembryo which had 6 prosuspensors had 23 or 24 in the embryo group, but this high figure was only found once, the next highest figure to it being 17, with 9 or 10 prosuspensors.

(C) *Early embryogeny.*—Reference to the dates given under "Explanation of Plates" shows that fertilization takes place about mid-June, and the stages so far described are completed in little over a week. Slow growth of the prosuspensors now begins, and the subsequent developments in the embryo cells, except for one small change, are not found till about mid-July when another bout of intense activity occurs which finally fashions the young embryo proper. About the time, however, that the prosuspensors first show coiling the embryo cells enter a peculiar phase. The cell contents tend to contract greatly in fixation, and the cytoplasm to stain even more intensely than before, so that the group of cells looks like a dark degenerating clump. Fig. 37 shows such a stage, which may be called the *resting* stage as it continues till elongation of the prosuspensors is almost complete. The cells gradually come out of the resting stage before the next phase supervenes; they fix better, the nuclei contrast more strongly with the cytoplasm, and nucleoli become very distinct. This is the appearance already referred to as shown in Fig. 43. The difference is quite obvious in most cases. The terminal cell begins to show signs of degeneration during the downward growth of the suspensor, and may be totally degenerate at the stage of Fig. 43, although its form may be retained for some time later, presumably due to the firmness of its wall. A couple of the embryo cells above it may occasionally degenerate also. It may be that this specialized cell facilitates the penetration of the embryo into the starch cone of the endosperm. Only in two other species in all the podocarps, and these also species of *Stachycarpus*—viz. *P. spicatus* and *P. ferrugineus*—does a similar cell seem to occur. In the former it also degenerates, but its fate in the latter is uncertain (Buchholz, 1936). In both cases it is described as containing two nuclei like those in the other embryo cells. A rechecking of this might be advisible.

From about this stage onwards embryos were dissected out whole as well as sectioned. As no cleavage took place later the dissected ones added little except confirmation to the knowledge gained from sectioning. A couple of photographs of dissected embryos are included. Fig 44 shows one photo-

graphed whole at the stage already shown in Fig. 43, although naturally nuclear detail is not obvious without special staining and special photographic technique.

In later embryos of podocarps the cells are known to be uninucleate. Buchholz (1936) suggested for *P. spicatus* that "cell plates appear between the two nuclei of each embryo initial or unit followed by other cell divisions," but no figures are given. Doyle and Looby (1939) made a similar statement for *Saxegothaea,* and supported it with a published figure. But material about this stage in *Saxegothaea* was scanty, and this figure was taken from *one* dissected embryo only, re-examination of which showed that the appearance might be open to other interpretation. In any case it is unsafe to draw conclusions from a single preparation, so particular attention was given to this stage in *P. andinus.* When this work was well advanced another paper by Buchholz (1941) came to hand. In this paper he figured a double division in *one* of the two embryo initials in *one* proembryo in *P. totarra,* the other embryo initial having become four-celled. The further statement is made that "no doubt the wall between two binucleate cells is delayed until after this pair of spindles has given rise to four nuclei." The meaning of this is not clear and perhaps confuses a further important statement in which he goes on to suggest that it is probable that in all podocarps the binucleate proembryo cells *directly* form four-celled groups. Actually this latter interpretation, put by Buchholz on his apparently isolated observation in *P. totarra,* is probably correct, as it is the condition also shown in *P. andinus.* The problem in this type is, however, a somewhat more difficult one. The point to be settled is whether a membrane forms between the two nuclei of a binucleate proembryo cell before the nuclei divide, or whether a simultaneous division of the two nuclei leads through a four-nucleate stage to a four-celled grouping. In *P. andinus* it is not a matter of following one or two embryo cells, but a group of cells very variable in number. An embryo with a large number of cells could be in quite a young stage or in an older stage in which divisions had taken place; and in whole dissected embryos in which divisions could be seen it was impossible, owing to the thickness of the embryo groups, to be satisfied with the location of the mitoses. Sectioned embryos, however, gave good results.

This phase—the change from the binucleate to the uninucleate condition of proembryo cells—is, in *P. andinus,* passed through rapidly after the embryo group recovers from the resting stage, and misinterpretation was fatally easy. At a certain stage in development, which may be called the *tetrad* stage, most of the embryo clump is seen to be composed of uninucleate cells compacted in four-celled groups. In each of the Figs. 52–54, which are sections from the same proembryo, at least one such group is obvious, these being the tetrads lying in the plane of the sections. The other tetrads are cut at various angles. Thus the cells grouped at *a* in Fig. 52 are probably not a single tetrad, but parts of two different ones. This tetrad stage could readily be recognized in dissected embryos, but was difficult to photograph, again owing to the thickness

of the whole embryo. In Fig. 56 the arrow points to a fairly distinct one, and in Fig. 57, from a partly crushed embryo, two tetrad groups can be picked out. Further, however, stages such as those in Figs. 45 and 46 were common about this phase. The first obvious interpretation of Fig. 45, showing two simultaneous mitoses in contiguous cells (flanked on the left by a binucleate cell), is a derivation from a binucleate cell in which a wall first formed between the nuclei followed by a division in each resulting cell, thus giving a tetrad. The lower group of cells in Fig. 46, from the edge of the *same* proembryo as Figs. 52–54, could be a similar case in which one of the cell pair, formed by the first membrane, lagged in its division a little behind the other. These are, however, in reality *post*-tetrad divisions.

A typical example of what actually occurs in *P. andinus* is shown in Figs. 47–51. These are serial sections from the same embryo, obviously in a young stage as some of the cells are still binucleate, Fig. 51. Fig. 47 shows on the right a cell in which two simultaneous divisions, indicated by the arrows, are taking place. The mitotic figures are not in the same plane, so only a little of the right-hand group of chromosomes appears. Figs. 48 and 49 are views of the next section in two different focal planes. The focus in Fig. 48 is on the fragments of the left mitosis in Fig. 47, the mitosis on the right being now more prominent. In Fig. 49 this division on the right is focussed, that on the left, owing to the lie of the blurred chromosomes in relation to the edge of the cell, giving the appearance of a faint nucleus. To the left above is a cell with a large nucleus in early prophase. A second nucleus in this cell appears in Fig. 50, which also shows a clear single division at the lower right. In Fig. 51, in the same position, another mitosis can be seen. These two divisions are almost certainly in the one cell, but when divisions are in different sections, as here, it is hardly possible to *prove* that they are in the same cell, still less to determine whether or not a membrane exists between them. Other, perhaps better, cells might have been selected instead of this series, but it seemed of greater interest to show what an average one of the smaller embryos actually looks like on the slide when completely sectioned.

From such double divisions the tetrads are formed, and in each of the Figs. 47–50 some stage in a tetrad grouping, much out of focus, can be seen. It is obviously of interest to determine how the membranes are formed between the resulting four nuclei, and the relations of these membranes to the old cell wall, but this has not yet been done. All the material available at stages such as these has been stained for chromosomes and nuclei. A further intense collecting and a restaining for pectic membranes will be necessary for a proper cytological examination, but a further reference to Figs. 54, 55, and 52 is of interest. The cell marked with an arrow in Figs. 54 and 55 (to the right of the very distinct tetrad) is also drawn in outline in Fig. 59. This cell is one shortly after the double division, but before membrane formation. It suggests that the pairs of nuclei are first separated by a cleavage plane in the position in which a membrane has been claimed to occur in *Saxegothaea* before division. Now in the one cell drawn and described by Buchholz (1941)

only one spindle lies in the plane of the figure, the other is almost at right angles to this plane and only an indefinite clump of chromosomes is shown, but a membrane is drawn developing across the single spindle visible. Of course such membranes, even if present between the nuclear pairs in Fig. 59, would not be visible owing to the lie of the cell, but the four nuclei are all in an advanced resting stage, and no trace of any spindle on which a membrane would develop could be detected either here or in any other case of a paired mitosis. It is possible that a cleavage membrane is formed here also. In the cell marked with an arrow in Fig. 52 and outlined in Fig. 58 four nuclei are present, and membrane formation is definitely in progress, but no spindle connections could be made out between the nuclei. However, until a large number of suitably stained intermediate stages are available, the question of membrane formation must remain uncertain.

In view of these data from *P. andinus* it is clear that, not only is Buchholz's (1941) interpretation of his single fortunate observation correct, but it is probable that this tetrad stage is fundamental in *Podocarpus,* since it occurs in one of the simplest forms with a large group of embryo cells, and in one of the most advanced species with only one or two such cells in the proembryo. Buchholz's older figure (1936, Fig. 9) probably shows this tetrad stage also in *P. spicatus.* It will be important to examine this stage in other genera. Older figures, again of Buchholz's (1933, Figs. 3, 7), almost certainly show this in *Dacrydium cupressinum* as well. Early revision of *Saxegothaea* is clearly essential. The tetrad stage may well prove an important family character like the binucleate condition in the young proembryo.

During the period of tetrad formation the cells of the embryo group enlarge a little, the whole group becoming looser and more bulged, as a comparison of Figs. 53 and 56 with Fig. 44 shows. There is, however, no embryo cleavage. Although it is easy to visualize how an increased independence of these tetrads could lead to the cleavage polyembryony of *Dacrydium* and some species of *Podocarpus*, yet in *P. andinus,* although hundreds of embryos at various stages were examined, not the faintest suggestion of cleavage or lobing was seen.

On completion of the tetrad stage divisions take place in the now uninucleate cells, the whole embryo becoming gradually more compact. Fig. 60, from a dissected embryo, shows a typical example just before the formation of embryonal tubes. For a short time, however, there seems to be a tendency for neighbouring cells derived from the same tetrad to divide more or less together, the divisions in Figs. 45 and 46 being of this type. As a result the developing embryo may show temporary tetrad-like groupings, or may appear as if faintly divided into sections, but the change from tetrad organization to that of a homogeneous embryo system is rapid. Figs. 69 and 70 are typical embryos with developing embryonal tubes. In the latter figure the tubes have only recently begun to elongate and are just pushing up the prosuspensors; in the former they are clearly much further developed. In both, the original terminal cap cell, which never enters into the organization of the embryo proper, can still be seen as a small pointed dark body at the tip.

The fleshy fruits, about the size of a cherry and pleasant to the taste, fall before the embryo is mature. On the oldest embryo met with at this stage the first development of cotyledons was just discernible. The same early fall was recorded for *Saxegothaea* (Doyle and Looby, 1939). This may be due to the trees growing outside their proper range, but Buchholz (1936) mentions a similar condition in seeds of *P. ferrugineus* gathered in New Zealand. It may be that an after-development of the embryo is another podocarpean feature. The phenomenon is worth closer investigation.

The endosperm in the meantime has been developing in a manner essentially similar to that already described for *Saxegothaea* (Doyle and Looby, 1939, Figs. 2C, D; pp. 131–2). The general appearance at a low magnification is shown here in Fig. 68 for comparison. The embryo in this section is that shown in Fig. 70.

The prosuspensor length varies considerably but averages about 6 mm.

(D) *Variations in embryogeny.*—These were strikingly few. Fig. 67 illustrates a condition found twice. This is a young proembryo in which the upper open-cell tier has not yet been cut off, and in which the embryo cells are still uninucleate. There are, however, at least 45 cells in the embryo group, 23 can be counted in this one section. These cases are almost certainly derived from an early stage in which a further free nuclear division took place before wall formation, giving 64 nuclei in all, which could clearly yield about 45 embryo cells, about 10 in an upper open tier, and a few relict nuclei. Rosette cells, at least in the sense used by Buchholz, were rare, appearing in not more than six embryos in four of which there were only one or two (Fig. 65). Fig. 66 is a diagram of a section from a type found twice. In view of the emphasis laid by Buchholz on the so-called rosette cells, such as those in Fig. 65, and of the fact that the cutting-off from the upper proembryo tier of a special open-cell tier seems an important phase in early podocarpean embryogeny, the origin of these smaller walled cells should be determined. That can hardly be expected from so stable a type as *P. andinus*, but may be attempted in some more variable species. It is possible that later divisions may occur in the prosuspensors, as one of these in the embryo from which Fig. 67 was taken showed a clear mitosis in metaphase.

(E) *Remarks.*—Any full discussion on development in the podocarps as a whole is postponed until work, at present in progress on *P. nivalis*, is completed, but a few points need further short comment here.

1. In only one other species from any genus of podocarps has the development of the proembryo and the early embryogeny been reported. This seems to have been done for *Podocarpus nagi* in a paper by Tahara (1941) which was not available and is only known from a short reference by Buchholz (1941). How much detail is presented in the paper or what wealth of material was available does therefore not appear. However, the proembryo is described as having 32 free nuclei before wall formation. These nuclei become organized into a single lower tier of 7–9 walled embryo cells and an upper tier of 23–25 cells open to the archegonium above. The upper tier is

described as forming, by more or less simultaneous division as in *P. andinus*, a prosuspensor tier proper and an open-cell tier which is not permanent. Whether or not true relict nuclei are extruded is not stated. Now in most cases the number of free nuclei that form the early proembryo in podocarps is not known accurately, and is judged from the number of prosuspensors and embryo cells that can be counted in older stages from dissected out embryos with the prosuspensors well grown. The numbers obtained are variable, but in most podocarps the number is probably sixteen. No proembryo has been accurately followed from the 16-nucleate stage, so that it is not known if any of the nuclei in such cases are extruded. Extrusion of a few nuclei might account for some of the variations in the numbers recorded, but in only four other species, in addition to the definite cases of *P. andinus* and *P. nagi*, is a 32-nucleate stage probable, judging from the large number of prosuspensors present. These species are *P. usumbarensis, P. gracilior, P. nankoensis* and possibly *P. blumei* (Buchholz, 1941, Tahara, 1941). It is of interest then that three of these species, *P. andinus, usumbarensis* and *gracilior*, belong to the sub-genus *Stachycarpus*, that the other three all belong to the section Nageia, and that, of these latter, two of them, *P. nagi* and *nankoensis*, belong to that sub-division of the section in which no fleshy receptacle is formed, the ovular arrangement resembling that in *Stachycarpus*.

2. The formation of an upper *open-cell tier* in *P. nagi* is also important. Coker (1902) suggested that such a tier was formed in the one species of Eupodocarpus studied by him, but had not enough evidence to be certain. Now that that stage is known to occur re-examination of Coker's figures clearly shows that such a tier was developed. Further in one of Buchholz's papers (1941) a figure is given (his Fig. 1) of a stage in *P. dacrydioides* with elongating prosuspensors above which are drawn a number of nuclei, referred to by him merely as relict nuclei, which must have had a similar origin. Thus in four sub-divisions of *Podocarpus*—the sub-genus *Stachycarpus* and the sections Nageia, Dacrycarpus and Eupodocarpus—the upper tier of embryonic nuclei divide to give an *open-cell tier* above the functioning prosuspensors. This condition must be considered just as fundamental in podocarpean embryogeny as the binucleate embryo cells proper. Thus immediately after primary membrane formation all the nuclei divide, roughly simultaneously, those below to form the binucleate cells of the embryo, and those above the uninucleate prosuspensor and open-cell tiers, the latter not being permanent. It is to be expected that a similar phase will be shown in the other genera of the family, certainly, at least, in *Dacrydium, Phyllocladus*, and *Saxegothaea*. Fig. 1A in Doyle and Looby's paper (1939) of the proembryo in the latter genus probably shows this phase, but the genus needs reinvestigation to make the point definite. In future descriptions of podocarpean proembryos a clean distinction must be kept between *relict* nuclei proper and the nuclei of this *open-cell tier*.

3. Within the sub-genus *Stachycarpus* two variations in gametophyte and proembryo exist. *P. andinus, spicatus*, and *ferrugineus* possess two (at most

three) long, pointed archegonia and a tiered group of embryo cells, like that here described for *P. andinus*, with a definite terminal cap cell. The proembryo in *P. ferrugineus*, and possibly in *P. spicatus*, is derived from a 16-nucleate phase. On the other hand, *P. gracilior* and *usumbarensis* have four or five shorter and more oval archegonia, and have a proembryo derived probably from a 32-nucleate stage, in which about ten embryo cells are formed lying in one tier. Before the data on *P. andinus* were available Buchholz (1936) suggested that the *P. usumbarensis* condition was the basal podocarpean type, and that the long archegonia, 16-nucleate phase, and tiered proembryo with cap cell, were derivative, the latter two features being causally related to the development of the long, pointed archegonia. The demonstration of a 32-nucleate, perhaps even in some cases a 64-nucleate, phase in *P. andinus* seems to make this view a strained one. Although suggesting that the *development* in *P. spicatus* and *ferrugineus* is derived Buchholz agrees with the general view that *morphologically* the *P. andinus-spicatus* type is simpler. If, then, development in *P. andinus* be taken as primitive a clear sequence follows. It has already been emphasized (Looby and Doyle, 1939, 1944) that the gametophytes of *P. andinus* (and probably also *P. spicatus* and *ferrugineus*) are almost identical with those of *Saxegothaea*, and at least closely resemble those of *Phyllocladus* and some species of *Dacrydium*. The common occurrence in these different types of the longish, pointed archegonia suggests that it is a retention of a basal form. In all also is the tiered proembryo with cap cell, except perhaps in *Dacrydium* species, which show certain signs of advance. The more direct view then would start with *P. andinus*. The primitive narrow pointed tip of the archegonia, with 32 or even 64 free nuclei, necessitated a rather coarse tiered proembryo, a condition continued in *Saxegothaea*, *Phyllocladus*, and some species of *Stachycarpus*, although in these the number of free nuclei may be reduced to 16. Within *Stachycarpus*, while retaining the 32-nucleate stage, a shortening of the archegonia and a widening of their base permitted the organization of the embryo cells into a single tier, a change continued directly into the Nageia section (see above). In the more advanced species of *Podocarpus* a greater precision was developed, the free nuclear number being kept at sixteen, the embryo group becoming more exact and specialized till ultimately reduced to a single cell, and the archegonia at the same time becoming more numerous and much shorter. A similar change though not quite so precise and specialized seems to characterize the genus *Dacrydium*.

4. Buchholz (1941), in his comments on the paper by Tahara (1941) already referred to, further suggests that Tahara has shown in *P. nagi*, for the first time in any podocarp, that the typical binucleate condition in the proembryo is derived from a division in each of the original cells, and not by an inclusion in pairs of the original nuclei. This, of course, must occur in *P. nagi* as in all other podocarps and could probably be seen to be fully illustrated were the paper available, but it seems necessary to point out that, two years earlier, Doyle and Looby (1939) had drawn a figure (their Fig. 1A) of *Saxegothaea* showing simultaneous divisions in what could only have been

uninucleate cells of the young proembryo. The caption under the figure ran "primary embryonic cells becoming binucleate" and it is quite clear that these authors were satisfied concerning the origin of the binucleate stage.

5. The lack of any cleavage polyembryony in the primitive embryo of *P. andinus* is further evidence in favour of the view that polyembryony is a derived condition. The form of the embryo at the tetrad stage is such, however, as to point directly to such advances as the lobing in *Saxegothaea* and the cleavage in *Dacrydium* and species of *Podocarpus.*

6. Finally it is suggested from a survey of the whole development in *P. andinus* that, in its gametophytes, proembryo, and early embryogeny, it may be taken as a prototype for the whole podocarpean complex, except for the inequality of the male cells which were probably primarily equal or subequal as they still are in the living *Saxegothaea* to-day.

Summary.

A discharge of archegonial cytoplasm between the neck cells precedes fertilization. Stages in the fusion of the male and female nuclei are given. The fusion nucleus divides *in situ,* the spindle being intranuclear. The second division, also intranuclear, occurs a short way down, the four nuclei migrating to the base where successive simultaneous divisions give 32 free nuclei, of which a number up to 10, called the *relict* nuclei are not included in the subsequent embryo organization by cleavage planes. This shows an upper tier of 9–13 cells open to the archegonium and a coarsely tiered group of 11–15 embryo cells with a large terminal cell. The upper tier divides to give a prosuspensor tier and a non-permanent *open-cell* tier. This tier is probably a fundamental feature of podocarpean embryogeny. The single nuclei of the other cells each divide to give the typical *binucleate* stage, which enters into a peculiar *resting* phase during prosuspensor elongation (6 mm.). Later, double divisions in each cell followed by membrane formation give rise to 4-celled groups throughout the embryo, the *tetrad* stage. With subsequent divisions the embryo becomes homogeneous and proceeds to further development without any cleavage polyembryony.

It is suggested from a survey of the whole life-history of *P. andinus* that, in its gametophytes, proembryo, and early embryogeny, it may be taken as a prototype for the whole podocarpean complex except for the inequality of the male cells which were probably primarily equal or subequal as in *Saxegothaea.*

EXPLANATION OF PLATES.

Plate 11.

1, 2. Cytoplasmic extrusion from archegonium before fertilization. 1 × 140, 2 × 300; June 13.

3. Male cell enters between necks. × 140; June 15.

4–7. Stages in fusion of male and female nuclei. 4 × 300; June 16. 5 × 300; June 19. 6 × 140; June 16. 7 × 140; June 15.

8. Sterile nuclei from pollen tube above fusion nucleus. × 300; June 19.
9–10. First sporophytic division. 9 × 140, 10 × 300; June 15.
11. The two nuclei passing down; remains of fusion nucleus above. × 140; June 16.
12–13. Second division. 12 × 300; June 16. 13 × 300; June 19.
14. Three of the nuclei just after telophase; location of fusion nucleus still visible above. × 140; June 19.
15. Three of the four nuclei passing to base of archegonium. × 140; June 22.
16–18. The four nuclei in the dense basal zone. × 140; June 22.

PLATE 12.

19, 20. Third division. × 140; June 22.
21–24. Sections from one proembryo to show the 8 nuclei. 21–23 × 300, 24 × 140; June 22.
25, 26. Fourth division. × 140; June 22.
27–29. Stages in 16-nucleate proembryo; 27, 28 from one embryo showing 9 of the nuclei in late telophase. × 300; June 22.
30. Stage in 32-nucleate proembryo; two *relict* nuclei above. × 300; June 22.
31. Proembryo cut tangentially to show four *relict* nuclei free above. × 300; June 22.
32, 33. Nuclear organization before cleavage. × 300; 32 June 19, 33 June 24.
34. Stage in formation of cleavage membranes. × 300; June 22.
35, 38. Young walled proembryo. × 300; June 22.
36. Uninucleate embryo cells. × 600. June 22.
37. Typical resting stage. × 300; July 12.
39. Formation of prosuspensors and *open-cell* tier. × 300; June 22.
40. Division in the uninucleate embryo cells giving the binucleate stage. × 600; June 22.
41. Proembryo with *open-cell* tier still intact. × 300; June 19.
42. Nuclear type before *resting* stage. × 600; June 22.

PLATE 13.

43. To show embryo nuclei after *resting* stage; degenerate cap cell below. × 600; July 12.
44. Complete dissected embryo at this stage. × 300; July 16.
45, 46. Post-tetrad divisions. × 300; 45 July 16, 46 July 20.
47–51. Serial sections of one embryo to show actual stages in paired divisions, etc., giving rise to *tetrads*. × 600; July 12.
52–54. Typical *tetrad* stage, all from one embryo. × 300; July 20.
55. To show embryo cells of 54, especially one with arrow, at higher mag. × 600; July 20.
56, 57. Dissected embryos to show tetrads. × 300; July 16.
58, 59. Intermediate stages in tetrad formation. 58 drawing × 1100 of marked cell in 52; 59 × 1250 = ditto of cell in 54 and 55, July 20.
60. Dissected embryo becoming homogeneous after tetrads, before embryonal tube formation. × 140; July 16.

PLATE 14.

61. Male and female nuclei in contact at fertilization. × 45; June 16.
62. Position of second sporophytic division. × 45; June 16.
63. Four embryonic nuclei at top of basal zone. × 140; June 22.
64. To show relative size of embryonic area to whole archegonium. × 45; June 22.
65. Irregular walled embryo. × 300; June 28.
66. Proembryo with two 'rosette' cells. × 300; June 22.
67. Proembryo probably from a 64-nucleate stage. × 300; June 22.
68. To show developing endosperm round young embryo with elongated prosuspensors. × 20; July 16.
69. Maturing embryo with embryonal tubes. × 300; July 20.
70. Embryo from 68, embryonal tubes forming and pushing up prosuspensors. × 300; July 16.

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No. 27.

A MOLECULAR CONSTANT FOR SOURED MILKS.

III.—Very Old Samples.

By J. J. RYAN,
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[Read June 27. Published July 25, 1944.]

In previous numbers of these proceedings (1), (2), (3), molecular constants suitable for the detection of watering have been worked out for fresh milk, for milk held for about three weeks without preservative, and for milk preserved with potassium dichromate and held for about two months.

The further problem of deciding the genuineness or otherwise of samples of unpreserved milk, which have been held for several months, is often met with. The present and final note deals briefly with the possibility of extending the method of molecular constants to such old samples. For this investigation the original freezing point of the milk has been compared with a cryoscopic constant derived from analytical determinations of the lactose, chloride, and total phosphate, in the soured milk. The convenient approximate determination of lactose by refractometry employed in earlier articles is unsuitable for such old milks. Instead, lactose is taken as the difference between the total solids determined by the method of the British Government Laboratory (4) and the sum of the fat, protein, and ash. Chloride is determined by the method outlined by Ryan and Pyne (1), and soluble phosphate by the method of Pyne (5), as modified for sour milk (2). End point difficulties, sometimes met with in the last estimation owing to the sour milk on neutralization becoming yellowish or brown, can be overcome by adding a few drops of potassium chromate solution to the magenta standard. Alternatively, both chloride and phosphate estimations can be made on the sour milk filtrate or the centrifugally separated serum.

For a milk containing L grams lactose, C grams of chloride (as NaCl), and P grams soluble phosphate (as P_2O_5) per 100 grams of water:—
Freezing point $\Delta = 0{\cdot}052\,L + 0{\cdot}615\,C + 0{\cdot}585\,P + a = K + a$, where a is the contribution of minor constituents and K the cryoscopic constant used here (1).

For the calculation of K the three percentages, lactose, chloride, and phosphate, must of course be corrected for the dry matter of the milk, and expressed as weights per 100 grams of water.

For nine samples of individual milks examined after holding for about three months at ordinary temperatures the values found for K varied from 0·443 to 0·466. When each value was compared with the original freezing

point of the corresponding milk determined by the Hortvet Cryoscope it was found that the sum of the lactose, chloride, and phosphate contributions furnished from 79 6 to 85 per cent., or on an overage 82·4 per cent., of the total depression (the real ratio is somewhat higher, as the formula for K is based on corrected freezing point depressions while the Hortvet values are uncorrected for supercooling, and average about 3 per cent. too high.)

Taking the average ratio obtained here, 82 4 per cent., and calculating on this basis the freezing points of the milks from the K values, figures are obtained which run from at most 0·018° C. higher to 0·019° C. lower than the actual freezing point as determined by the Hortvet.

H. J. Evans (6) has described a method of determining the original freezing point of soured milk which bears some resemblance to the present one. He dissolves the amount of lactose in the fresh milk (determined by the method of the British Government Laboratory) in the appropriate volume of a solution prepared from the ash in such a way that the calcium and phosphorus are thought to exist in a similar condition (as far as their effect on the freezing point is concerned) to that of the original milk. The freezing point of the artificial solution is then determined. The values thus obtained were found to differ from the original freezing points by amounts ranging from + 0·008° C. to - 0 019° C., differences comparable with these obtained here, using the molecular constant.

For the latter the maximum departure of the calculated freezing points from the values experimentally obtained on the fresh milks is about 3 per cent. By the use of either the molecular constant K or, what amounts to the same thing, the freezing point calculated from it, it should be possible therefore to estimate added water in very old samples of milk with an accuracy of about 3 per cent. This is probably as high a degree of accuracy as can be expected, taking into account the difficulties attending the determination of the original lactose, much of which will usually have undergone considerable fermentation by the time the analysis is made.

Summary.

A molecular constant for old soured samples of milk, based on corrected lactose, chloride, and phosphate allows of the determination of added water with an accuracy of about 3 per cent.

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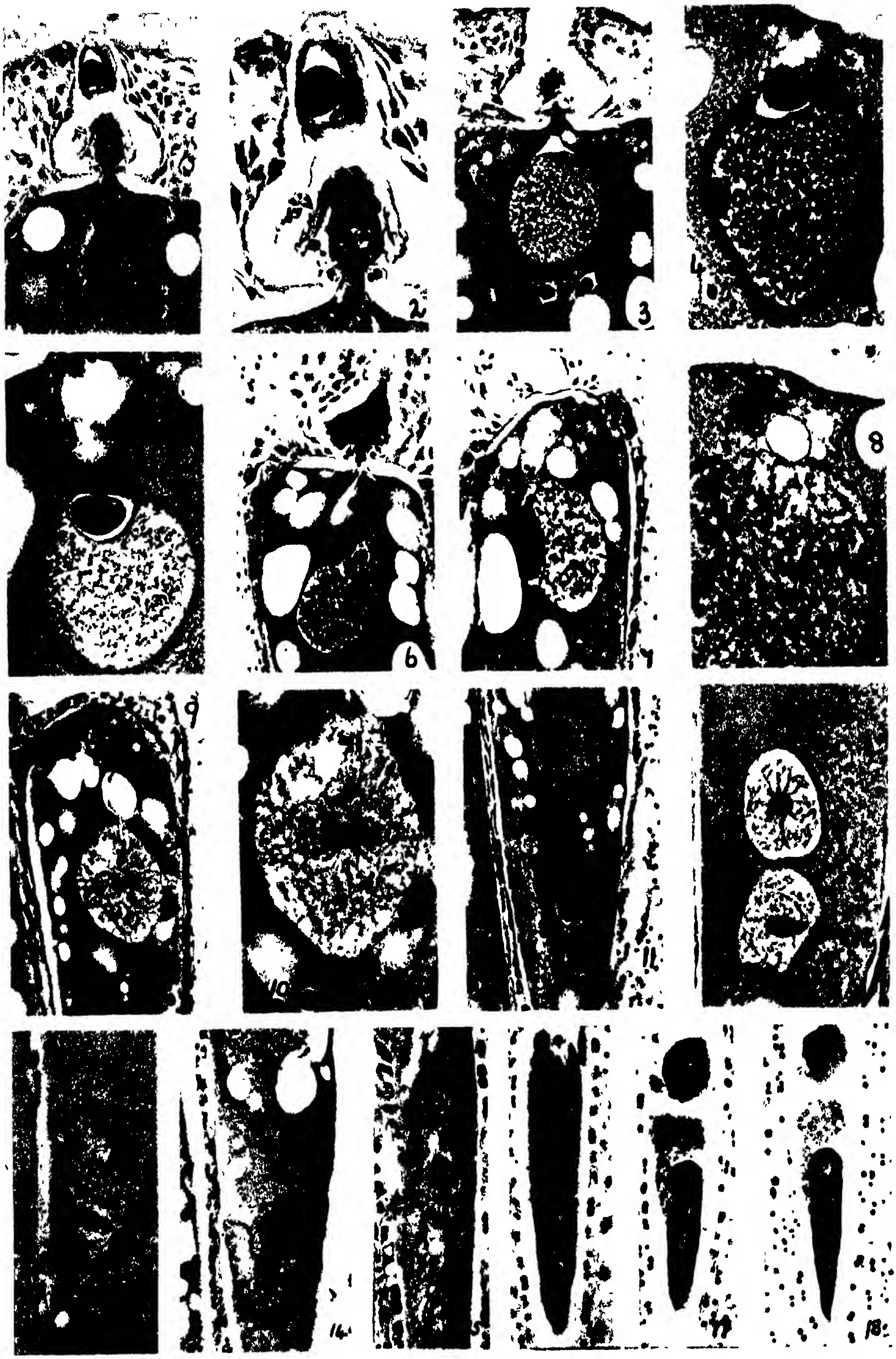
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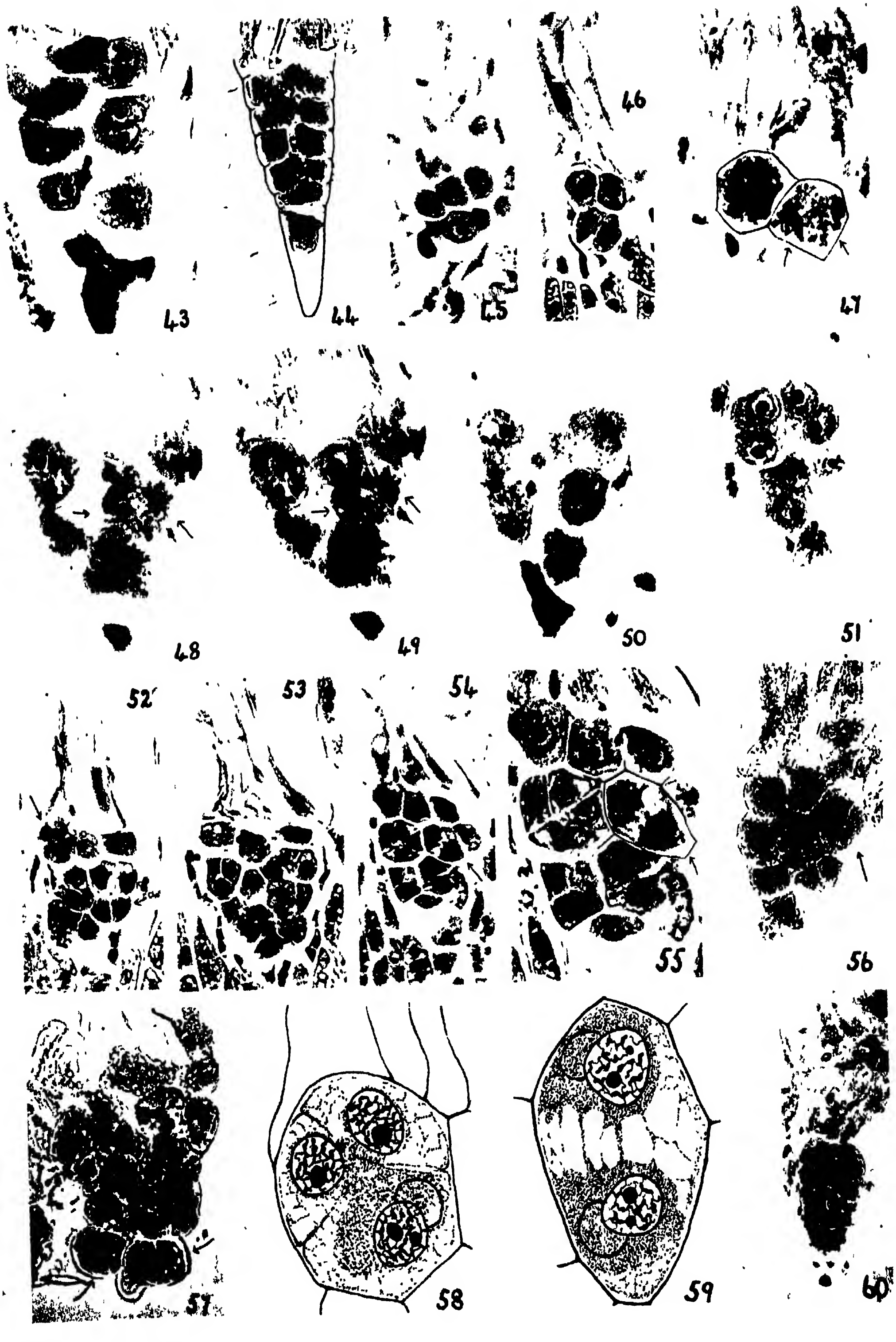
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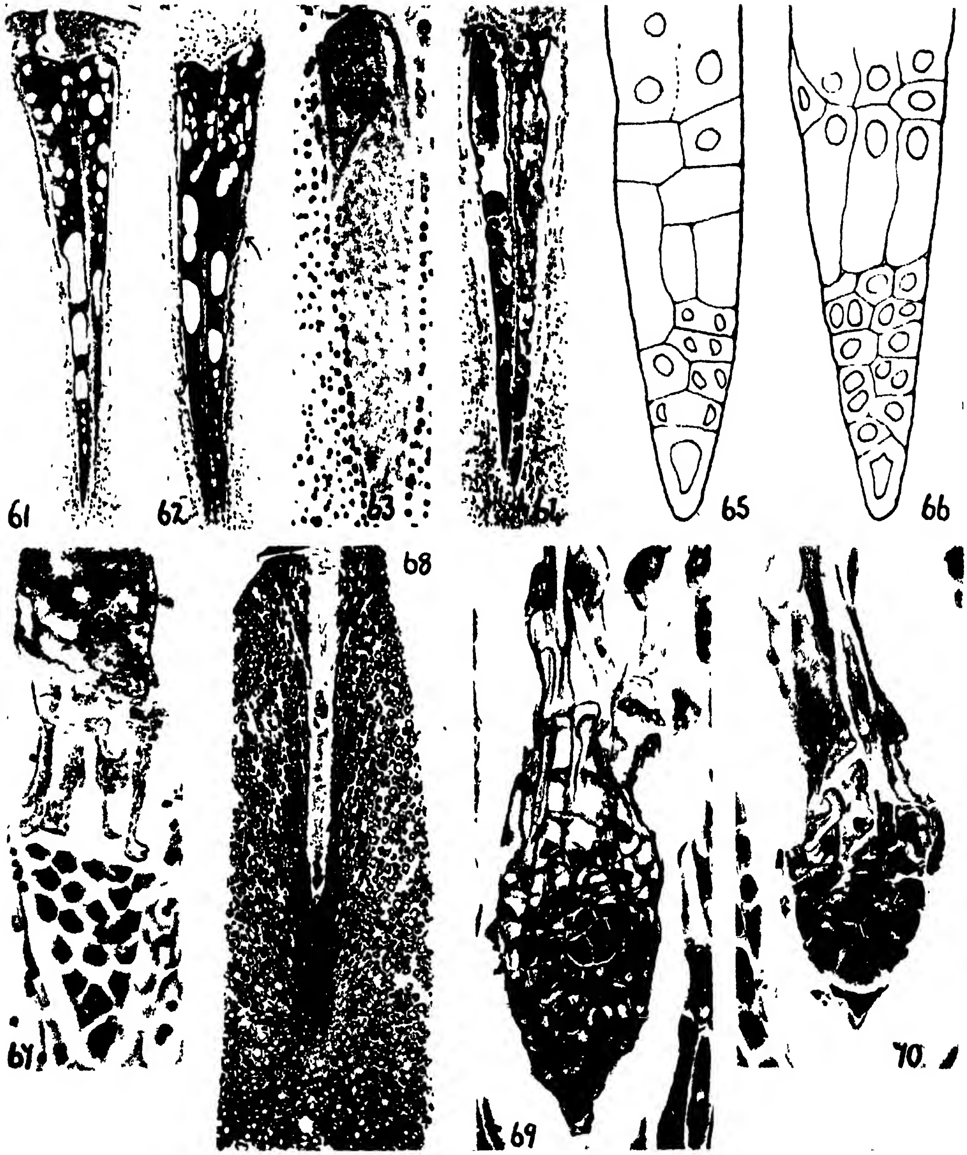
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No. 28. AUGUST, 1944.

OBSERVATIONS ON A SEVERE STRAIN OF POTATO VIRUS X.

BY PHYLLIS E. M. CLINCH,
Albert Agricultural College, Glasnevin, Dublin.

(PLATES 15–17.)

No. 28.

OBSERVATIONS ON A SEVERE STRAIN OF POTATO VIRUS X.

By PHYLLIS E. M. CLINCH,
Albert Agricultural College, Glasnevin, Dublin.

(PLATES 15–17.)

[Read FEBRUARY 22. Published separately AUGUST 11, 1944.]

THE potato virus now generally referred to as X (*Solanum Virus* 1, *Marmor dubium*), is probably the most widespread of those affecting potato stocks. In America it was found to be a universal constituent of the principal commercial varieties; and while many European varieties are at least partly free from X, others such as Up-to-Date and Duke of York are almost invariably infected.

It has been recognised for several years that different strains of X are in existence. Johnson (17) and Koch (19) distinguished between the "mottle" and "ringspot" forms of latent mosaic, the names being descriptive of the type of symptom produced on inoculation to tobacco. Köhler (23) made a similar distinction in the case of X viruses occurring in field potatoes in Germany, adding that each form contained strains of varying virulence. Murphy and McKay (26) observed that a latent top-necrosis virus was widespread in American material examined by them, and they equated it with the top-necrosis virus commonly carried by Up-to-Date (virus B). Recently, Clinch (9) showed that the Up-to-Date virus is a strain of X, readily distinguishable from other strains by its reactions in certain varieties of potato. Salaman (29) recognises six different strains of virus X. These vary from an extremely mild form X^{11}, which is almost symptomless in potato, *Datura stramonium*, and tobacco, to the strains designated X^{S} and X^{N}, which cause severe symptoms in these hosts. Salaman showed that these various strains of X were mutually protective.

In this country, the commonest form of X disease in commercial seed potato stocks is one causing a mild or almost imperceptible mottling of the foliage of affected plants. Murphy and McKay (26) used the term simple mosaic in reference to this disease, which they distinguished from other potato mosaic diseases by its effects on different varieties of potato. Clinch and Loughnane (10) observed that the symptoms evoked in tobacco and other solanaceous hosts by inoculation of simple mosaic from different potato sources were not identical. The form which they referred to as mosaic *ex* C produced in tobacco a definite pattern of chlorotic rings and necrotic spots, while the symptoms of the standard simple mosaic from the laboratory stock were more nebulous although of similar type. Similarly, in other solanaceous hosts the

two differed, but only in the intensity of the symptoms produced, except in intolerant varieties of potato such as Arran Crest and Epicure, in which the reactions are identical. Both would now be defined as mild forms of X. Koch and Johnson (20), who afterwards examined the same material, identified the milder form as "mottle" and the stronger one as "mottle and ringspot," a diagnosis which will be discussed later.

Because of the prevalence of simple mosaic, it had come to be more or less accepted that severe mosaic in the field was invariably due to mixtures of simple mosaic with other viruses, such as A and Y. However, it is now recognised that a strain of X exists in this country which, alone, is capable of causing a severe disease in potatoes. It was first encountered by the writer in 1934 in a single plant of a small crop of British Queen potatoes grown from certified seed in Co. Dublin. The writer's colleague, Dr. R. McKay, now believes that the same disease was present in crops inspected by him several years previously, and that it was probably diagnosed as crinkle at that time. In 1938 another colleague, Mr. J. B. Loughnane, observed it in seed potatoes stocks in Co. Donegal, and samples were also received from the Midlands and other centres. Mr. Loughnane and Mr. P. Keenan, Inspector in the Department of Agriculture, reported that only a very small percentage of infected plants occurred in any one crop, and that in some cases the plants appeared to be only partially infected. It was at first believed that the disease was a composite one, but inoculation studies failed to reveal an underlying virus other than X.

The present paper is an account of the symptoms and behaviour of this virulent strain. In order to avoid complications the same virus stock was used for all the reactions about to be described, fresh inoculations to virus-free potatoes being carried out each year.

Symptoms of Severe X in Potato Varieties.

The symptoms produced in 32 varieties of potato have been observed both in house-grown and field plants. Infection has been conveyed by sap inoculation, top-graft, and tuber core-graft, and is readily effected irrespective of the technique employed. Excluding Arran Crest, Epicure, and King Edward, which react with top-necrosis, all the varieties tested react in a more or less similar manner. The following is an account of the typical reaction produced in young, actively growing virus-free plants following sap inoculation by the rubbing method:—

Local necrotic lesions appear on the inoculated leaves after 6–10 days; these are circular, blackish, and small at first, but increase in size very rapidly and coalesce, so that large areas of the leaves wilt and turn grey. Eventually, these leaves wither and drop off. About 10–14 days after the appearance of local lesions systemic symptoms begin and develop with great rapidity. In the sub-apical leaves the veins clear, and pale blotches may appear, spreading

outwards from the main veins; then, suddenly, all the subsidiary veins become necrotic, black streaks appear on the undersides of the large veins, and the leaves collapse and wither in a short time. Superficial brown streaks may also occur on the stems and petioles. Thus the initial phase of the systemic disease is a distinctly necrotic one, and the leaves affected at the onset of secondary symptoms either drop off at once or dry out and remain hanging for a time (Fig. 1). The foliage which develops subsequently shows conspicuous symptoms of a different type. The leaves are severely puckered and rugose, and display a brilliant mosaic mottling as well as numerous dry, interveinal necrotic lesions. They are extremely brittle, and attain little more than half the normal size (Fig. 2). As a consequence of the stunting, chlorosis, and severity, of the symptoms generally, a variety may be rendered almost unrecognisable in the field. The tubers appear normal, but the yield is considerably reduced.

During the phase of veinal streaking, especially when the latter is aggravated by dry conditions, the symptom picture resembles that of primary Y infection, but the subsequent mosaic mottling, which persists during the life of the plant, clearly distinguishes virus X. (The upper leaves of Y-infected plants are rugose, with a characteristic sheen but without a distinctive mosaic pattern). Even more readily might the secondary mottle symptoms of severe X infection be confused with the disease known as crinkle, due to X + A (25). Excluding the leaf drop and more widespread necrosis, which in the first year of infection serve to distinguish severe X from crinkle, the symptoms of the two diseases are very similar, those of severe X being the more conspicuous in Arran Banner, Majestic, and most other varieties.

In the second year of infection the leaf-drop symptom is absent. Otherwise the plants appear as in the first year of infection. Growth is stunted, the plants being about half the normal size; the leaves are severely puckered, and display a brilliant mosaic mottling together with numerous "rusty" necrotic lesions in the interveinal areas (Fig. 6).

The following varieties react in the manner described above:—Arran Banner, A. Cairn, A. Consul, A. Peak, A. Pilot, A. Signet, A. Victory, Ballydoon, British Queen, Catriona, Champion, Di Vernon, Doon Early, Doon Star, Dunbar Cavalier, Dunbar Rover, Dunbar Yeoman, Eclipse, Gladstone, Great Scot, Kerr's Pink, Majestic, President, Redskin, Shamrock, Sharpe's Express, Ulster Monarch.

Dunbar Standard was less severely affected than other varieties. The lower leaves did not drop, nor was the vigour of the plants so seriously impaired. The variety Irish Chieftain, which carries virus A, reacted with a severe crinkle ((25) (10)) accompanied by dropping of the lower leaves and interveinal necrosis.

The effect on the varieties Arran Crest, Epicure, and King Edward was exactly similar to that caused by milder strains of X. Following grafting, all three reacted with top-necrosis, and the "eyes" of the tubers were killed.

Sap inoculation resulted in local necrotic lesions, rarely followed by systemic necrosis and death of the plant.

Plants grown from tubers grafted with diseased cores showed systemic symptoms similar to those following sap inoculation (Fig. 2). Usually only one or two of the shoots showed infection on coming above ground, and sometimes all shoots appeared normal at first. Beginning in one or two shoots, the disease gradually spread to others until the entire plant was infected. The initial symptoms invariably took the form of a necrosis of all the fine veins in the sub-apical leaves. This led to leaf-drop accompanied by the mosaic symptoms, already described. If, as occasionally happened, a shoot had attained a height of 10–12 ins. before becoming infected, it failed to show the typical symptoms, the leaf drop and severe distortion being absent.

Introduction of Severe X to plants already infected with Mild X.

As expected, the previous presence in a potato plant of a mild strain of X completely prevented the introduction of the severe strain by sap inoculation. When the grafting method was employed, however, the severe strain was enabled to effect an entry. Following core-grafting of severe X into 9 President tubers infected with simple mosaic, only one plant showed the puckering and rusty necrosis symptomatic of severe X in some of its shoots. The plant was only slightly stunted as compared with control plants infected with severe X alone; and, with continued growth, the upper parts of the foliage showed only a mild mottle

Three President plants infected with mild X were then top-grafted with severe X scions. After about 3 weeks the stocks showed, in addition to the mild mosaic mottle, some rather conspicuous yellow blotches with necrotic centres. Although the symptoms were not comparable in severity with those of control plants having severe X only, it was obvious that the severe strain had been introduced, but appeared to have difficulty in becoming established. On cutting the tops of the plants, however, the new shoots which developed showed more or less typical severe X symptoms, and when sap from them was inoculated to *Datura stramonium*, the full symptoms of severe X were produced. Six tubers from plants infected with the mixed strains were grown in the field in the following year. In most of the resultant plants the majority of the shoots showed typical severe X symptoms on coming above ground, the remaining shoots showing a mild mosaic; but, as the plants developed, the upper leaves of the severely infected shoots flattened out and showed only the mottle symptoms of the milder strain. Consequently, by the end of June, when the plants were approaching maximum size, they had quite a distinct appearance from plants in an adjoining drill which were infected with the severe strain only, and which exhibited fully severe symptoms throughout the season.

SYMPTOMS IN SOLANACEOUS HOSTS OTHER THAN POTATO.

Datura stramonium.—The inoculated leaves develop circular necrotic lesions after 4–6 days. These increase rapidly in size, and the leaves soon wither and drop off. Systemic symptoms appear in the upper leaves about the 9th day. The veins clear and rapidly become necrotic, so that large areas of the leaves (usually the distal portions) wilt and then dry out (Fig. 7). The remaining areas show ruffling and distortion and a brilliant mosaic pattern of chlorotic rings and blotches interspersed with numerous "rusty" brown necrotic lesions. Growth is severely checked with the onset of symptoms but continues later, the new leaves displaying conspicuous mosaic symptoms accompanied by a lesser amount of necrosis. The leaves generally are much reduced in size, and the plant, as a whole, severely stunted. The flowers are also affected, developing grey or brownish patches on the calyces and corollas, usually resulting in premature withering. The virus is not carried in the seed from infected plants.

Nicotiana glutinosa.—After 4 days the inoculated leaves display small circular grey spots which rapidly develop into dark zoned lesions about 5 mm. or more in diameter; these coalesce and dry out, whereupon the leaves wither and drop off. Meanwhile, about the 9th day, symptoms appear in the young leaves at the top of the plant. The veins turn yellow, then rapidly become necrotic, particularly in the distal portions of the leaves, which soon wilt and dry out. The remaining areas are ruffled and distorted, and display a brilliant yellow mosaic pattern interspersed with numerous rusty necrotic lesions, symptoms which continue to appear in all subsequent leaves. The plant is severely stunted, and the effects generally are extremely severe (Fig. 8).

Lycopersicum esculentum (Tomato).—The inoculated leaves develop necrotic rings or circular blackish lesions accompanied by other necrotic configurations associated with the veins. Secondary symptoms appear in the top of the plant 9–10 days after inoculation; the leaves curl downwards, the veins clear, then small greyish patches develop all through the interveinal areas. These spread rapidly and coalesce, whereupon the leaves collapse and wither. As new leaves unfold they display similar symptoms, except that the wilting is less severe. Interveinal necrosis remains the dominant feature of the symptom picture (Fig. 5), being sometimes preceded by a conspicuous yellow mosaic in the young growth. The lesions usually appear first in the terminal leaflet, then spread to the remaining leaflets. They may be circular or irregular in shape, and, according as growth is "hard" or "soft," they may be dry and reddish-brown in appearance or grey and water-soaked, respectively. In the latter case they spread more rapidly, and the leaves soon wither. No lesions were observed on the stems or fruit.

Tomato plants inoculated with a mixture of severe X and tobacco mosaic (*Nicotiana Virus* 1) were killed by a virulent necrotic disease within 14 days after inoculation. When milder strains of X (simple mosaic and mosaic *ex* C)

were substituted for the severe strain, the symptoms were correspondingly milder, consisting of more or less extensive necrotic lesions on the leaves and petioles, and streaking of the main stem also in the case of mosaic *ex* C. The actual growing points, however, survived.

Solanum nodiflorum.—Local lesions are numerous and consist of purple-fringed red rings with chlorotic centres; the inoculated leaves rapidly turn yellow and drop off. Secondary symptoms appear in the top leaves after 10 days; the veins develop a conspicuous yellow colour, and their development appears to be inhibited. The interveinal areas are ruffled and distorted, and are covered by dark reddish markings, many of which are ring-shaped. The necrotic lesions enclosed by them turn yellow, and the leaves drop. Within a few weeks only a few leaves at the top of the plant remain. These are puckered and distorted, and display a conspicuous yellowish green mottle accompanied by dry necrotic lesions. The plant as a whole is severely stunted.

Solanum nigrum.—Reacts very similarly to *S. nodiflorum.* Primary symptoms take the form of circular black lesions which increase rapidly in size; the intervening tissue turns yellow, and the leaves drop off. Secondary symptoms develop in the top leaves, the veins becoming yellow at first and then almost necrotic; the interveinal areas show puckering and severe chlorosis together with dry necrotic lesions. Affected leaves fall at the slightest touch, and eventually only a few leaves at the top of the plant remain. Many of the blossoms drop prematurely, and the plant as a whole is severely stunted.

Capsicum annuum.—The inoculated leaves develop severe zoned necrotic lesions, and then drop off. Systemic symptoms appear first in the top leaves, the veins of which become severely chlorotic; numerous necrotic rings and angular markings develop in the interveinal areas, and the tops of young plants may be killed. If the tops survive the leaves are puckered and distorted, and show pronounced mottling accompanied by irregularly shaped necrotic lesions.

Nicotiana tabacum (Tobacco) *var.* White Burley.—About 4 days after infection the inoculated leaves show circular pale yellow areas, which tend to develop necrotic outlines. Systemic symptoms appear in the upper leaves about 10 days after inoculation and take the form of a conspicuous mosaic, the leaves being covered by innumerable small chlorotic rings and circular areas, usually outlined by bands of reddish or necrotic tissue or having a central necrotic spot. As new leaves develop they in turn display a conspicuous mosaic, which begins as a yellow spotting at the tip of each newly unfolded leaf. The growth of the plant as a whole is stunted. The severe strain was recovered unchanged from White Burley plants which had been held in the greenhouse for 6 months (October–April) after inoculation.

Nicotiana tabacum (Tobacco) *var.* Orinoco.—Circular blackish necrotic lesions develop on the inoculated leaves after 3–5 days; these increase rapidly in size, and coalesce, and in a short time the leaves wither and fall off.

Usually there are no further symptoms in this variety, but secondary symptoms occasionally develop. Vein clearing in the top leaves may be followed by the development of chlorotic rings and spots which rapidly become necrotic; but more often the secondary symptoms are exclusively necrotic, consisting of necrotic rings, circular spots, and streaks, all of which are associated originally with the veins. The rings increase in size or become surrounded by larger rings which may coalesce, so that large areas of the leaf wither. The disease advances acropetally, but the necrotic reaction in the leaves apparently serves to hinder the multiplication of the virus, so that invariably the upper portion of the flowering shoot appears healthy and may in fact be virus-free. In the plant shown in Fig. 9, photographed seven weeks after inoculation, virus X could not be demonstrated in the three healthy appearing leaves immediately above the diseased ones, nor in the symptomless half of the uppermost diseased leaf. All these areas responded with local necrotic lesions to severe X inoculum, besides which discs of tissue previously removed from them failed to produce symptoms on inoculation to *Datura*. When the tip of the plant was cut off, three axillary shoots which developed immediately below the point of severance appeared healthy; and when removed and rooted separately they produced virus-free plants. In the meantime, the parent plant produced further axillary shoots on the lower part of the stem, which in course of time showed necrotic symptoms once more.

NON-SOLANACEOUS HOSTS OF VIRUS X.

Hosts of virus X outside the family *Solanaceae* are almost unknown. Jones *et al.* (18) recorded *Amaranthus retroflexus* L. as a host of latent mosaic. Salaman (29) experimented with plants of twelve different families and recorded systemic infection only in two, *viz.* chrysanthemum and *Browallia speciosa*. The latter he classifies as a member of the *Scrophulariaceae*, but Willis (32) and other authorities include this exotic species in the *Solanaceae*. Of the remaining plants a small number, including red beet and sugar beet (*Chenopodiaceae*) and horse beans (*Leguminosae*), reacted with local necrotic lesions.

In the present work a number of common weeds from the potato fields and surrounding hedges at Glasnevin were inoculated with severe X sap. The results obtained were as follows:—

Lamium hybridum (*Labiatae*).—This host becomes infected fairly readily, although the percentage of successful infections is not so high as in solanaceous hosts. After about 10–14 days, pale-yellow or light-brown lesions usually appear on the inoculated leaves. They may be more or less rounded at first, but later the outlines become diffuse and they develop into irregularly-shaped dark rusty patches. Systemic symptoms consist of a diffuse mottling accompanied by light-brown or reddish-rusty lesions, the latter occurring

mainly on the intermediate leaves of the plant. Growth is stunted and the infected plants have a sickly appearance. The symptoms produced by simple mosaic (10) were of the same type but distinctly milder. Both strains were recovered unchanged on *Datura* and *N. glutinosa*. This appears to be the first record of systemic X infection of a non-solanaceous weed native to these islands.

Veronica agrestis (*Scrophulariaceae*).—Circular, black local lesions began to appear after 10–12 days, and reached a diameter of about 4 mm. There was no systemic infection.

Beetroot and Mangold (*Chenopodiaceae*).—Six days after inoculation small circular lesions appear all over the rubbed leaves. These coalesce and the affected leaves wither and drop; there are no systemic symptoms. Simple mosaic induces similar lesions, but they are fewer in number and slower in developing.

No infection was secured in the following plants: Caryophyllaceae: *Stellaria media*. Chenopodiaceae: *Chenopodium album*. Compositae: *Carduus arvensis, Chrysanthemum indicum, Senecio vulgaris, Sonchus oleraceous, Taraxicum officinale, Tussilago farfara*. Convolvulaceae: *Convolvulus arvensis*. Cruciferae: *Brassica oleracea, Capsella bursa pastoris*. Fumariaceae: *Fumaria officinalis*. Labiatae: *Galeopsis tetrahit, Salvia splendens, Stachys sylvatica*. Papavaraceae: *Papaver rhoeas*. Plantaginaceae: *Plantago lanceolate*. Polygonaceae: *Polygonum aviculare, Rumex obtusifolius*. Ranunculaceae: *Ranunculus repens*. Rosaceae: *Potentilla anserina*. Rubiaceae: *Galium apperine*. Scrophulariaceae: *Antirrhinum majus*.

Physical Properties of Severe X.

Results of repeated tests with freshly-expressed sap showed that the severe strain was similar in its physical properties to the common mild strains of X. These results were as follows:—

Thermal inactivation point.—68° C. (10 mins. exposure).

Filterability.—Sap was highly infective after passage through L 1, L 3, and L 5 grades of Pasteur-Chamberland filter candles.

Longevity in vitro.—At 17°–20° C. there was a progressive decrease in infectivity of crude sap up to approximately 28 days, when inactivation was usually complete. Crude sap held at 5° C. was still infective after 60 days; the period necessary for complete inactivation at this temperature was not determined.

Resistance to dilution.—Infection was obtained at a dilution of 1 in 50,000 but not at 1 in 100,000.

Serological Experiments.

In the course of some work on the serological reactions of potato viruses, tests were made both with the severe and mild (mosaic *ex* C) strains of virus X,

the results of which have a bearing on the present subject. The immune sera were obtained from rabbits, and the immunising antigens were the freshly extracted juices of potato foliage diluted 1 in 5 with water and clarified by filtration. The injections, six in number, were given intraveneously at 3–4 day intervals, the doses being 0 5, 1 0, 1 5, 2·0, 2 0, 2 0 c.cs. Ten days after the last injection the animals were bled, and the serum obtained was centrifuged and stored in the frozen state. To prepare the test antigens expressed juice was diluted slightly, then twice frozen overnight, thawed, and centrifuged, when a clear light-brown supernatant liquid was obtained.

In carrying out flocculation tests, 2 c.c. of antigen was mixed with 1 c.c. of serum, serial dilutions being made in 0 85 per cent. saline. The mixtures were incubated at 37° C. for 30 min., then held at 5° C. overnight to allow precipitates to settle. Healthy plant sap was tested at the same time as virus extract with the usual controls of sap and saline and sap and normal serum. The sera were not absorbed beforehand, as precipitates due to healthy potato proteins and their antibodies were slight, they did not occur at all when the test antigens were prepared from *Datura stramonium*.

The results of the tests showed that both strains of X induced an antiserum of high titre following a single course of injections as described above; in fact, blood probes indicated that active serum could be obtained even after the 4th injection. The high serological activity of virus X has already been demonstrated by Chester (6), Spooner and Bawden (31), and others. Incidentally, it may be mentioned that the writer failed consistently to obtain a serum active towards potato virus A, even after three courses of injections, while the reactions obtained with anti-Y sera were weak and unsatisfactory (cf. Gratia and Manil (15) and Hansen (16)). This applies to tests with green saps according to Chester's (8) method, as well as to those with cleared antigens.

In these experiments no significant difference was detected in the comparative ability of the severe and mild strains of X to induce antibody formation. The sera of both reacted at approximately the same dilutions; if anything, the anti-severe X sera were slightly more active than the anti-mild X sera; but considering the method of preparation of the antigens and the probable differences in reaction of individual rabbits, no distinction could be made between the two strains in this respect.

Several tests were made with anti-X sera and extracts of X-infected and healthy tubers. The tissues were macerated with a little distilled water, and allowed to stand for 3 hours at room temperature. The juice was then filtered through muslin, centrifuged, placed in a cold chamber (5° C.) overnight, and again centrifuged. Separate extracts of the skin and flesh of the tubers and of areas surrounding and including the dormant buds, were prepared; but contrary to the results of Gratia and Manil (15), none of these extracts flocculated with immune serum. Extracts of diseased roots which were prepared in the same manner showed a reaction, but this was much weaker than that

obtained with leaf extracts. One concludes from these results that the virus is less concentrated in the roots than in the leaves, and that the quantity present in dormant tubers is extremely small. Confirming the latter view is the fact that only a very low percentage of infection was obtained in inoculation experiments with the juice from infected tubers. Other workers, e.g. Burnett and Jones (5) and Johnson (17) experienced the same difficulty in securing infection with tuber extracts.

Attenuation of the Severe X disease in Potato.

It has been mentioned that the first sample of severe X examined by the writer occurred in a crop of British Queen plants grown in Co. Dublin. Inoculations were made from the diseased plant to virus-free British Queen, and the stock thus infected was maintained in the usual way by tuber propagation in the glasshouse. In the 3rd year of infection an attenuation in symptoms was observed, which persisted in the following year. In the 5th year of infection all traces of the virulent disease seemed to have disappeared from the stock, which, when grafted on ten other varieties of potato, produced only a mild disease of the simple mosaic ((26) (10)) type.

In view of this behaviour a careful examination was made of the reactions of different varieties of potato to severe X over a period of 3–4 years. The virus used was isolated from field plants in Co. Donegal, and appeared to be identical in every respect with that isolated in Co. Dublin. Certain varieties were inoculated in the glasshouse, and propagated subsequently both in the glasshouse and in the field. These included Arran Banner, Arran Cairn, Arran Consul, Arran Signet, Ballydoon, Champion, Di Vernon, Doon Early, Kerr's Pink, and President. Other varieties were inoculated in the field, and the infected stocks kept under observation for four successive generations. These included Arran Peak, Arran Pilot, Arran Victory, British Queen, Catriona, Dunbar Standard, Dunbar Yeoman, Great Scot, Gladstone, Majestic, Redskin, Shamrock, and Ulster Monarch. The course of the disease was essentially similar in all cases. In the first year of infection the plants showed severe mosaic, puckering and necrosis of the foliage, and leaf drop; in the 2nd year similar symptoms but without the leaf drop. During this period, inoculation to other solanaceous hosts resulted in the typical severe X symptoms. In the 3rd year of infection an appreciable change took place. The plants, on coming above ground, showed the fully severe symptoms of the previous year, but as growth continued the new leaves appeared free from puckering and necroses, and exhibited only a relatively mild mosaic mottle (Fig. 3). The symptoms produced on *Datura* by inoculation from the upper and lower leaves of such plants were quite distinct; in the latter case conspicuous local necrotic lesions were followed by the systemic symptoms typical of the severe strain; in the former case local lesions were absent or inconspicuous, stunting was

negligible, and the mosaic symptoms corresponded to those of an X virus of mild or medium intensity (Fig. 7). Sometimes the merging of the severe disease into the mild form was marked by transient intermediate symptoms of conspicuous yellow blotches on otherwise green leaves. Inoculation to *Datura* showed that the severe strain was present in the chlorotic areas, a mixture of the severe and mild strains in the tissues immediately adjoining them, while elsewhere only the mild strain was present.

By the 4th year of infection the potato plants were entirely free from the puckering and necrosis characteristic of the severe strain. Symptoms were reduced to a yellowish blotchy mottle on the lower leaves, and even this was extremely mild in some units. The degree of stunting was negligible, and a striking contrast was afforded by the appearance of two plants, one in the 2nd and the other in the 4th year of infection growing side by side in the field (Fig. 6). So far as could be judged from mass inoculation to *Datura*, the foliage of the recovered plants was free from the severe strain and contained only a virus of the common mild X type. However, as separate inoculations from finely dissected portions of the mottled leaves were not undertaken, the possible existence of the severe strain in minute areas of these leaves cannot be ruled out.

This reduction in virulence occurred consistently irrespective of variety or whether the plants were grown in the glasshouse or in the field. Although the transformation usually occurred in the manner described above some variations in this procedure were observed. For example, one plant of Kerr's Pink grown in the glasshouse showed attenuation of symptoms in one shoot in the 2nd year of infection, while the remaining shoots were fully severe throughout that season (Fig. 4). Of six plants grown from tubers of this unit in the following year four were infected with severe mosaic and two with a milder mosaic, and inoculations from them to *Datura* resulted in severe and mild symptoms, respectively. Eventually all units of the clone recovered. This phenomenon of premature recovery in isolated shoots was also observed in field grown plants of Kerr's Pink and other varieties. Occasionally a batch of plants showed general recovery in the tops of the shoots in the 2nd year of infection; in such cases a mild strain or, more often, a mixture of the severe and mild strains of X could be isolated from the apical growth; the succeeding generations of such plants generally showed the intermediate yellow blotch symptoms, already described, in their lower leaves. On the other hand, there were instances of individual plants of certain clones failing to show initial symptoms of recovery until the end of the 4th year of infection, although the remainder of the stocks showed attenuation in the previous season. Generally speaking, however, the transformation from the severe to the mild X disease was complete in the 4th year of infection.

It is possible that different environmental conditions may hasten or retard the diminution in virulence of the severe X disease. This view is

suggested by the fact that a group of field plants which showed milder symptoms as early as the 2nd year of infection had been subjected to warm, dry weather during the period of most active growth. Furthermore, it has been observed that the effect of bright sunlight is to cause a lessening in intensity of the mosaic symptoms, although the fact that severe symptoms develop with the advent of duller conditions indicates that this is mainly (but perhaps not entirely) a masking effect. While it cannot be stated with certainty that warm dry conditions facilitate the transformation from the severe to the mild disease, there is a certain amount of evidence pointing in this direction. On the other hand, the phenomenon of premature recovery in isolated shoots suggests control by internal, rather than external, factors. So far no evidence has been obtained that the subjection of infected tubers to high or low temperatures affects the virulence of the disease symptoms in the subsequent plants; and in fact the alteration seems invariably to occur in the foliage during the growing season. It was observed that the enforced production of new growth at the transitional period served to extend the period of predominance of the severe strain. Thus, on cutting back in the 3rd year of infection a young President plant whose lower leaves showed the virulent disease, the axillary shoots which were stimulated to growth showed the full symptoms of the severe strain, and continued to do so almost to the end of the season. Corresponding plants which had been allowed to grow naturally produced mildly infected foliage throughout the same period, so that the amount of severely diseased foliage was much greater on the cut than on the uncut plants at the end of the season.

Although certain factors may possibly affect the rate of transformation from the severe to the mild disease, the occurrence of the change seems to be inevitable; and having once attained the mild form the disease appears to become stabilized. So far no alteration in the reverse direction has been observed, and attempts to reproduce severe symptoms in fully recovered plants by cutting the tops or by growing them under different environmental conditions have been unsuccessful. It cannot be said that the strain of mild X which replaces the severe strain is identical in every plant or clone. The symptoms in the recovered potato plants vary, as already stated, from a clear blotchy mosaic to an almost imperceptible mottle; and such variations may be found between clones of the same variety infected at different times. There is a corresponding difference in the symptoms produced in *Datura* by inoculation from recovered plants, but this difference, both in potato and in *Datura*, is one of degree and not of kind. In general, the strains present in the recovered plants would correspond to the simple mosaic and mosaic *ex* C already mentioned. The severe strain may be said to differ in kind, as well as in degree, from such forms by reason of its characteristic symptoms of necrosis, distortion, and severe stunting.

EXPERIMENTS ON THE MOVEMENT OF SEVERE X IN U.S.D.A. SEEDLING 41956.

Numerous attempts to introduce the severe strain to 41956 by grafting or by sap inoculation with the aid of carborundum powder were unsuccessful. The 41956 plants failed to show symptoms, and were proved to be virus free by inoculation to *Datura* and by grafting on healthy Arran Crest and President, as well as on President containing mild X; no alteration of symptoms was effected in the latter. This result, besides confirming the statements of other investigators regarding the immunity of 41956 to X, also helped to dispose of an early idea that the severe disease might be due to a mixture of X with another virus, the latter being responsible for the necrotic symptoms.

It has been mentioned in a previous paper (9), however, that when 41956 was inserted as an intermediate scion in double-grafted plants, virus X was able to pass from an upper infected scion through the 41956 into a susceptible basal stock. The latter showed the typical X symptoms, but the 41956 remained normal in appearance, and attempts to demonstrate the presence of any virus in its foliage were unsuccessful. It appeared, therefore, that the virus had passed through the 41956 without multiplying therein. The time taken between the final grafting and the development of symptoms in the basal stocks of these double-grafted plants was of the same order as in single-grafted plants. Several experiments were carried out in this manner, all of which gave similar results.

Experiments were then made to determine whether virus X could travel upwards through 41956 with the same facility as downwards. Arran Banner plants were used as the basal stocks in two experiments. They were grafted, when 8–10 ins. high, with scions of 41956, which were allowed to grow at least 8 ins. in length before being top-grafted with scions of healthy Arran Banner. Control plants were similarly double-grafted, except that a healthy susceptible variety was substituted for 41956 as the intermediate scion. The plants were grown in 10 in. pots, and received liberal manuring during the season. Most of the top scions grew at least 10–12 ins. in length, and the intermediate scions and basal stocks were extremely vigorous.

In the first experiment the six Arran Banner basal stocks were originally healthy and were not inoculated with the severe X virus until the top scions were fully established; this was on June 9th, nearly 3 months after planting. After 14–18 days mosaic symptoms began to appear in the leaves of the basal stocks. Early in July the top scions and the shoots of the basal stocks were cut back to induce new growth. About 10 days later symptoms appeared in the intermediate scions of the controls, and after a further 7 days in the top scions of the same plants. Of the three plants having intermediate scions of 41956, however, none became infected with virus X in the upper scion up to the time of lifting on September 4th. The 41956 scions, therefore, had apparently served to prevent the passage of X from the basal stocks into the upper scions.

A second experiment was carried out in exactly the same manner except that the basal stocks were grown from X-infected tubers. The object of this was to save the incubation period following inoculation, and thus effect the introduction of the virus to the scions at an earlier stage. Both scions were established in from 2 to 2½ months after planting. Altogether 14 plants were grafted as described above, 10 with intermediate scions of 41956, and 4 controls with intermediate scions of healthy Arran Banner. The latter developed symptoms of severe X in 14–27 days after grafting, so that they were already systemically infected before the top scions were affixed; the top scions of these plants also showed symptoms in due course after they had become established. Of the 10 experimental plants with intermediate scions of 41956, the severe X reached the top scions in only two cases, although the plants were kept growing for 2 months after the final grafting. During the course of the experiment shoots were taken from the 41956 portions and grafted on Arran Crest potato, but even in those plants of which the top scions, as well as the basal stocks, were showing symptoms of severe X, the presence of the virus in the intermediate 41956 was not demonstrated.

In a third experiment 5 plants of President infected with a combination of viruses X and F (11) were grafted with 41956. In all cases the mild yellow spotting symptomatic of virus F appeared in the 41956 scions after 3–4 weeks. Top scions of healthy President were then affixed, two of which failed to become established. Of the three which grew actively, two became infected with virus F only, and the remaining one with both viruses.

These results show that the insertion of an intermediate scion of 41956 usually constitutes a barrier to the ascent of virus X in pot-grown potato plants over two months old. No such barrier is effected in similar plants by an intermediate scion of an X-susceptible variety; nor does 41956 affect the ascent of a virus to which it is susceptible, e.g. virus F. The ability to move upwards in such plants appears, therefore, to be bound up with the ability to multiply in the tissues to be traversed. Since movement of virus X through 41956 must be passive (being independent of multiplication), there is thus evidence of two types of movement of virus X in potato stems, the one passive and the other dependent upon multiplication.

In the case of the tobacco mosaic virus, which has been extensively studied in this connection, it has been established that the movement of the virus is intimately bound up with that of elaborated food materials, and the conclusion has been reached that the main path of rapid movement is in the phloem, through which the virus is transported mechanically in the food stream. A slow cell-to-cell movement is also envisaged, but differences of opinion exist regarding the actual mechanism involved in this process. It seems likely that this concept of the movement of tobacco mosaic virus is also applicable to virus X. There already have been indications of a correlation between the movement of X and that of elaborated sap (9), and the present results furnish

additional evidence of this relationship. In a plant such as the potato, which forms underground storage organs and does not normally set seed, the main flow of metabolites would be increasingly in a downward direction from the time tuber formation had been initiated. Cutting the tops of the plants with consequent production of new axillary shoots, would cause movement of food materials towards these new centres of utilization, but might not necessarily induce a complete reversal of flow if tuber formation were active. In the writer's experiments the passive movement of X, as exemplified by that occurring through 41956, took place freely and rapidly in a downward direction in pot plants which must have been in a state of active tuber formation, since they were about 2½ months old by the time union of scions was effected. Passive movement in an upward direction, however, occurred rarely in plants of this age, and failed altogether to take place in plants at a more advanced stage of growth. On the other hand, the type of movement which is apparently bound up with multiplication took place in an upward direction irrespective of the age of the plants, so that the direction of such movement can obviously be counter to the main flow of metabolites. A movement involving multiplication would presumably exclude the xylem vessels, if not the sieve tubes; hence the parenchymatous tissues are the most likely path of travel in this case. This movement is probably of the slow cell-to-cell type mentioned in regard to tobacco mosaic virus, although in the case of the latter virus it has not been shown that such movement is necessarily dependent upon multiplication.

The inability to demonstrate virus X in shoots from 41956 scions which the virus has obviously traversed may be due to a low concentration of virus in the scions. Bennett (4) has pointed out that the concentration of mosaic viruses in phloem is probably low, and it seems likely that virus X is confined to the phloem in the variety 41956. If the number of virus particles in the 41956 scion at any given time were small, the securing of infection by grafting shoots therefrom on healthy plants would obviously be fortuitous.

SPREAD OF SEVERE X IN THE FIELD.

It has not so far been shown that any insect vector is implicated in the spread of virus X in the field. Schultz and his co-workers (30) found that X-free potato seedlings contracted latent mosaic when grown in contact with infected plants or when brushed with diseased foliage; but the possibility of insect transmission could not be excluded in their experiments. Loughnane and Murphy (24) proved, however, that spread of X (simple mosaic) resulted from contact between the foliage of healthy and diseased plants grown in a glasshouse free at least from the larger sucking insects. They also showed that infection resulted from contact between the haulms of healthy and

X-infected plants in the field, although proportionately only about half as many plants (14 per cent.) contracted infection in the field as in the glasshouse. Infection was not contracted as a result of root contact only.

Experiments on the spread of severe X in the field were carried out during the three consecutive seasons 1940–42. The plants were grown in an isolated plot situated amongst beans or wheat and about 500 yds. away from other experimental potato plots. The location of the plot in the field was altered each year. The drills were 30 ins. apart and the plants 18 ins. apart in the drills. The plot was hand-worked and the chances of infection due to other than natural causes were reduced to a minimum. Majestic was selected as the principal indicator variety, and all the experimental plants used were derived from the same virus-free stock. Tests were also made with the intolerant varieties, Arran Crest and Epicure.

1940 Experiment.—The 1st drill in the plot contained 12 healthy Majestic plants, and the adjoining drill 12 plants of healthy Arran Crest. Next came a drill of Epicure, and then in the 4th drill 12 plants of Arran Banner infected with severe X. The first drill of Majestic was therefore situated at a distance of 7½ ft. from disease sources. Twelve more healthy Majestic plants were grown in contact with infected sources, the diseased and healthy plants being situated alternately in the drills. Twelve plants of healthy Arran Crest were also grown in contact with diseased plants in the same way. The remainder of the plot was occupied by plants of different varieties infected with severe X, excepting one drill in which were grown 12 plants of President infected with a mild strain of X. The latter was included for the purpose of observing whether any change occurred in the intensity of the mild disease.

The tubers were planted on 4th May. On 14th June, when the plants were 12–15 ins. high and growing vigorously, sap from each of the 24 Majestic plants was tested for X by inoculation to *Datura* and by means of precipitin tests with anti-X rabbit serum; the results were negative. They were again tested on 2nd July when the leaves of adjacent plants were first beginning to touch, but proved to be virus-free. By the middle of July there was generous contact between the haulms of adjacent plants, and a few weeks later the plants of adjacent drills were touching. On 9th August the Majestic plants were tested once more by inoculation from the upper leaves to *Datura* with negative results. On this data none of the test plants of Majestic, Arran Crest, or Epicure showed systemic symptoms of severe X, nor were any such symptoms observed up to the time the plants matured. The crop was lifted on 17th September, and the entire tuber yield of each test plant was saved, the units being stored separately.

In the following year all tubers were planted in the field, and examined carefully for disease symptoms. It was found that not a single plant derived from the 12 units of Majestic which were not in contact with severe X sources

had contracted infection; nor had any infection occurred in the units of Epicure and Arran Crest, irrespective of whether they were in contact with diseased plants or not. On the other hand 8 of the 12 Majestic plants which had been grown alternately in the drills with diseased plants had become infected—but in no case had the virus reached every tuber of the infected plant. On an average, 3 tubers out of the average total yield of 11 carried infection. Furthermore, in the majority of cases apparently healthy and diseased shoots came from the same tuber. The manner of appearance of the disease was similar to that occurring when tubers were infected by core graft; i.e. the initial symptoms consisted of blackish necroses on the leaves and streaking of the veins, the severe mosaic symptoms developing subsequently. It was quite common to find, in the middle of June, 4 shoots of a plant fully infected with severe X, the remaining shoots being apparently healthy; a month later the initial symptoms of veinal streaking and interveinal necrosis would have appeared in the previously healthy shoots, and eventually the entire plant would be affected. When initially healthy shoots became infected in this manner through spread of virus from other shoots on the same tuber, the fully developed basal leaves were the last to show infection, and appeared healthy for a considerable time after symptoms were visible in the tops of the shoots.

1941 Experiment.—The layout of this experiment was similar to that of 1940, but the number of test plants was increased. One drill of 15 healthy Majestic plants was grown at a distance of 7½ ft. from the sources of disease, while another drill was grown alongside a drill of infected plants. In addition, 15 Majestic plants were grown in the same drills as severe X plants, the diseased and healthy plants alternating in the drills. Fifteen plants each of the intolerant varieties Epicure and Arran Crest were also grown, being separated by a distance of one drill from diseased plants.

Owing to extremely dry soil conditions, the plants did not attain their usual size, growth was "hard," and the plants matured early. Consequently the degree of contact between plants of adjoining drills was less than in the previous year. The leaves of adjacent plants in the same drill began to touch about 8th July, and were in close contact by the end of July. During August a single shoot on each of 2 Majestic plants in contact with diseased plants was observed to show necrotic lesions and streaks on the leaves, and inoculation to *Datura* confirmed the presence of severe X in these shoots. Apart from these two cases, however, no symptoms were observed in any of the test plants of Majestic, Arran Crest, or Epicure. When the tubers of the experimental plants were grown in the following year it was found that only those Majestic plants which had been in contact with diseased sources in the drill had contracted infection, thus confirming the results of 1940 in every respect. The actual number of contact plants infected was 7 out of 15, i.e. about 47 per cent.

1942 Experiment.—In this experiment, which was similar to the preceding

ones, it was considered unnecessary to grow Majestic plants in contact with diseased sources, as the spread of virus between adjacent healthy and diseased plants in the same drill appeared to have been amply confirmed. Twenty-four healthy plants of Majestic were, therefore, grown at a distance of 5 ft. from a drill of diseased plants, while 12 healthy plants of Arran Crest were grown in contact with diseased plants. All these plants remained healthy in appearance throughout the growing season, and on 26th August the Majestic plants were tested for virus X, with negative results. When the tubers of the experimental plants were grown in the following year, it was found that not a single unit had become infected.

The conclusions to be drawn from these experiments are that in the case of a tolerant variety such as Majestic, severe X is transmitted freely by contact between adjoining healthy and diseased plants. Taking into consideration the results of Loughnane and Murphy (24) it may be assumed that transmission is effected through the leaves rather than through the roots of the plants. As no transmission whatever took place between diseased and healthy plants which were not actually touching, even though the distance separating them was at most 7½ ft., it may be concluded that under Glasnevin conditions insects are not operative in the spread of this virus. In the 3 years during which the experiments were in progress, thrips were abundant on the potato plants, being especially numerous in 1941. Aphids, although not plentiful, were present each year, and were sufficiently numerous to effect spread of leaf roll and virus Y in nearby plots on the same farm. Leaf hoppers were also observed on the potato plants in each of the three seasons. The results indicate, therefore, that these insects may be ruled out as possible vectors of severe X.

The absence of infection in the intolerant varieties Arran Crest and Epicure, even when grown in contact with diseased plants, is in accordance with the general experience that plants which react to a virus with top-necrosis do not contract infection with that virus in the field, due, it is believed, to the destruction of cells at the site of inoculation as a result of which movement of virus is restricted.

It is interesting to note that, although 67 per cent. of the "contact" Majestic plants actually contracted infection in 1940, none showed systemic disease symptoms in that year; and in 1941, when 47 per cent. infection occurred, close inspection revealed only the initial symptoms of the disease, and then only in a single shoot of each of two infected plants. In view of the severity of the reaction of Majestic to severe X, it is most unlikely that the virus was systemic in the foliage of those plants or shoots which remained healthy in appearance. All such foliage tested proved in fact to be virus-free. Hence the virus reached the tubers in the majority of cases without systemically infecting the foliage. In explanation of this it may be recalled that the leaves of adjacent plants were not in contact until the month of July, by which time the plants were almost fully developed and in a state of active tuber formation.

Presumably then the direction of major food transport was towards the tubers at the time infection took place. If it be true that the main movement of virus is with the food stream, it is not difficult to see why under the circumstances the virus should be carried from the site of inoculation on the leaf to the tuber without necessarily spreading to other parts of the plant.

The failure to become systemic in the foliage may also account for the frequency with which the tubers from the field-infected plants produced healthy as well as diseased shoots. Such a phenomenon has not been observed in the case of tubers from plants with diseased foliage. It is generally believed that the mosaic viruses multiply chiefly in the leaves, and it has been shown that downward movement of virus in a potato stem is not dependent upon multiplication. It may be suggested, therefore, that in the plants under discussion there was probably slight multiplication of virus at the site of inoculation until the vascular tissue was reached, and that the quantity of virus thus formed was carried to the tubers in the food stream without further reproduction. The amount of virus reaching the tubers of late-infected plants might therefore be extremely small. Furthermore, the results of inoculation and serological tests with expressed juice of tubers from plants systemically infected with X show that the quantity of virus in tuber juice is much less than that in leaf sap; which suggests that there may be little or no multiplication of virus X in the tubers. If these suppositions be correct, it is only to be expected that the quantity of virus in the tubers of late-infected plants would be insufficient to cause infection of every developing sprout. Only when an infected sprout formed green leaves might the virus multiply freely, and from such a source other shoots on the same tuber would eventually become infected, provided there was continuity of sap flow between them.

REFERENCES IN THE LITERATURE TO STRAINS OF X SIMILAR TO THE SEVERE STRAIN UNDER INVESTIGATION.

The X^N strain which Salaman (29) described in 1938, and which occurred originally in plants of the variety Majestic grown in Scotland, corresponds closely to the writer's severe strain in its symptoms on several varieties of potato and on other solanaceous hosts, as well as in its physical properties. Furthermore, referring to X^N in potato Salaman observed that "when a stock is carried on by tuber in the glasshouse it is not rare to find that in subsequent years in one or other plant of a clone a more or less complete clinical recovery has taken place. In such a case only X^G may be found." This passing reference indicates that the phenomenon of recovery is associated with X^N as well as with the strain described in the present paper.

There are certain points, however, upon which the writer's severe X differs from X^N as described by Salaman (29). For example, X^N was reported to be

less readily communicable by grafting than by sap inoculation, infection sometimes failing even in cases where union between scion and stock was good. Such has not been the experience in grafting experiments with severe X. It was also stated that X^N-infected tubers of Majestic and other varieties might in the second season give rise to healthy plants, fully infected ones, or fail to sprout. In the case of severe X, tubers from systemically infected plants invariably gave rise to diseased plants, and sprouting was not affected. Furthermore, leaf drop is predominantly a 1st year symptom of the writer's strain, while Salaman describes it as a conspicuous 2nd year symptom in the case of X^N.

In certain varieties of potato the symptoms produced by X^N differed from those of severe X. For example, Arran Banner, Ballydoon, and Doon Star, were stated to react with a relatively mild mottle; but the results were based on infection of only one experimental plant in each case. The reaction of Eclipse to X^N was found to be variable, this variety responding either with a mild interveinal mottling or else behaving as a carrier. It was believed, also, that mutation of X^N to X^S occurred during passage through Eclipse. These experiments, however, were confused by the fact that part, at least, of the clone of Eclipse used was already infected with a latent strain of X. Virus-free Eclipse (12 plants) infected with severe X in the present work reacted in the same manner as other tolerant varieties. With regard to intolerant varieties, Salaman found that the reaction of King Edward to X^N differed from that of Arran Crest and Epicure. No symptoms followed sap inoculation, but 2 plants behaved as carriers. Only a small percentage of plants was infected by grafting, but these reacted with top-necrosis. It was also suggested that X^N was converted into a strain of lower virulence by passage through this variety. In the writer's experiments King Edward, Arran Crest, and Epicure all reacted similarly to severe X.

The differences outlined above, if established, would be sufficient to distinguish the two strains of X. It seems likely, however, that some of them, at least, would be eliminated if the two strains were compared simultaneously under identical conditions.

Bald and Pugsley (1) reported that a necrotic strain of X, which they equated with Salaman's X^N, is sometimes a component of mixtures of X carried in Australian commercial potato stocks. It is also possible that similar strains occur in American varieties of potato, judging by the symptoms produced by certain of the American severe ringspot and "virulent latent" strains of X in tobacco, tomato, and other solanaceous hosts. The same may be said of strains isolated by Köhler ((21) (23)) from German potato stocks. Exact comparison is difficult without knowing the reactions of these various strains on appropriate virus-free varieties of potato.

The potato ringspot virus described by Putnam (28), which Salaman equates with X^N, also approaches the writer's severe X strain in its symptoms

on various non-potato solanaceous hosts as well as in its physical properties. In the variety President, however, Putnam's virus failed to produce symptoms; and unless the plants used were, like Green Mountain and Bliss Triumph, already infected with latent X, this reaction would constitute a clear distinction between his virus and both the X^N and severe X strains.

The D strain of virus X described by Bawden (3) resembles the writer's severe strain in its symptoms on a certain number of potato varieties. It causes top-necrosis, however, in Arran Consul and Majestic, is carried by Arran Banner, Champion, and other varieties, and also differs from the writer's strain in its symptoms on *Datura stramonium*, tomato, and *Nicotiana glutinosa*.

DISCUSSION.

The most interesting feature of the severe X disease described in the present paper is the consistent manner in which its symptoms become attenuated in affected potato stocks during the course of a few years. This recovery is distinct from the recovery of *Nicotiana* spp. from Tobacco Ringspot recorded by Price (27), in which the plants become carriers of the virus.

Two suggestions may be put forward to explain the reduction in virulence of the severe X disease: (1) That the original inoculum contains a mixture of severe and mild strains and that the mild strain multiplies with greater rapidity than the severe one and gradually displaces it from the stock; and (2) that the severe strain mutates to the mild form within the tissues of the potato plant. The chief difficulty in accepting the former explanation is that there is no evidence that the severe strain multiplies or moves through its hosts less rapidly than the milder strains. On the contrary, it is highly infectious and becomes systemic in potato and other hosts with a rapidity at least equal to, if not greater than, that of mild X. Bald and White (2) consider that the ability to multiply more rapidly than milder strains is an attribute of severe strains of potato virus X, although they visualise the possibility that a necrotic strain may limit itself by killing the sites for its own multiplication. In the disease under discussion, however, the most severe necrosis of the foliage occurs in the first year of infection, whereas recovery does not take place before the end of the second or third year. This indicates that the damage done to the tissues is not accountable for the limitation of this particular strain in potato. There are other reasons, too, for doubting that the attenuation is due simply to the settling out of a mild strain from an original mixture of mild and severe X. In Orinoco tobacco the severe strain tends to be strictly localized, whereas milder strains become systemic and produce mosaic symptoms. If the inoculum contained a mixture of mild and severe strains, a segregation thereof might reasonably be expected in this host, even allowing that a certain amount of destruction of a mild strain might occur in the lesions produced by the

severe form. No indication of the presence of a mild strain was obtained in this way. Furthermore, it has already been mentioned that when the severe strain was introduced by graft to potato plants infected with mild X, the plants to which the tubers gave rise in the following year differed in appearance from control plants infected with the severe strain only.

These considerations have led to the tentative conclusion that the recovery of potato stocks from the severe disease is not due simply to the sorting out of an original mixture of viruses, but results from the mutation of the severe strain to a mild form. Salaman (29) hesitated to invoke mutation in explanation of the recovery which he observed in X^N-infected potato plants in the glasshouse, suggesting that the change was due to the over-running of the plants by a mild strain of X present in admixture with the severe strain from the start. This view, however, is not in keeping with his experimental evidence, which indicated that X^N occurred originally in potatoes in the pure state. Apart from this, it is clearly difficult to explain the presence of mixtures of X strains in field plants except on the basis of mutation. So far as is known, the various strains of X are mutually protective, for Köhler's (22) statement that the mottle strains do not protect against the ringspot forms has been contradicted by Salaman (29) and also conflicts with the results obtained in this laboratory. The only known method of spread of virus X is by contact between diseased and healthy plants which is equivalent to sap inoculation under artificial conditions. Theoretically, then, a plant already infected with one strain of X should not become infected with a second strain under field conditions. Salaman (29) suggested that field plants infected with mixed strains might have received such strains either simultaneously, in a single inoculation, or in two or more infections at short intervals of each other; but this theory obviously implies the eixstence of a vector of virus X, of which there is at present no evidence.

That plant viruses do mutate is now generally accepted, but in the majority of cases recorded the altered strain has been localized, usually in small spots on the leaves. Wholesale changes are unusual and are certainly not a feature of mild X diseases such as simple mosaic and mosaic *ex* C (10), both of which have been cultivated at Glasnevin in many successive generations of potato without displaying any permanent general change in symptoms. The severe strain of X thus differs markedly from the common mild strains in its instability in potato. This consideration leads one to speculate regarding the origin of the severe strain. As there is no evidence of spontaneous generation of a virus the simplest theory would be that it arises by mutation within the potato plant from a milder strain and multiplies to the virtual exclusion of the latter. This view, however, is not supported by evidence obtained in the present investigation, for as yet no case of reversion from the mild to the severe form has been observed. Furthermore, Salaman (29) records that all strain reversions observed by him were from the more to the less virulent form and

efforts to increase the virulence of mild strains artificially were unsuccessful. There is also the fact, which is possibly significant, that the severe strain cropped up on several occasions in seed potato stocks raised originally from virus-free tubers and which might reasonably be expected to be more or less free from X (12). In the circumstances, one might go so far as to suggest that the severe strain is not native, as such, to the potato, but arises by passage of a milder strain through another host resulting in intensification; and that it tends naturally to revert to the mild form within the potato environment. It is true that there is no evidence that passage through solanaceous weeds alters the character of the severe strain; besides, such weeds are uncommon in Irish potato fields. The fact, however, that *Lamium hybridum* can be systemically infected suggests that there may be other non-solanaceous hosts of X which are as yet undiscovered and which might have an intensifying effect on mild strains of this virus.

Salaman (29) was the first to attempt a survey of the various strains of X occurring in potatoes in England. The strains are defined by their reactions on a variety of solanaceous hosts, principally the tobacco variety White Burley and *Datura stramonium*. Three of the six strains which Salaman describes, viz. X^H, X^N, and X^D, were found in the pure state in potato; the remaining three, X^G, X^L, and X^S, were isolated by subjecting the virus from infected potatoes to selective inoculation through several generations of tobacco, in the belief that the plants contained mixtures of strains. The drawback of this method of analysis is that it neglects the possibility of mutation within the tissues of the tobacco. If the development of new strains in isolated spots on tobacco leaves were to occur, in the case of virus X, with the same frequency as in the case of the tobacco mosaic virus, there would be no certainty that the strains isolated by this technique occurred as such in the original potato. Nevertheless, no better method is at present available, and Salaman states that one, at least, of the strains isolated in this manner (X^S) was also isolated direct from potato; while another of them (X^G) corresponds closely with the simple mosaic (10) common in potatoes in this country.

The grouping of X strains into mottle and ringspot forms according to symptoms on tobacco, originally adopted in America, is not sufficiently discriminating. It may even be unsound, for a strain of X from this laboratory, diagnosed as "mottle" by Koch and Johnson (20), is capable of inducing mild ring formation in tobacco under suitable environmental conditions, which suggests that there is no hard and fast line between mottle and ringspot symptoms in this host. Regarding the other basis of distinction between the two groups, viz. thermal death point, it is doubtful whether the end points obtained in experiments with crude saps are sufficiently sharp to render a difference of 2° C. a reliable diagnostic feature. It is not even certain that such a difference exists, as Salaman (29) records a thermal death point of 68° C, for the G and L strains of X which he equates with Koch's (19)

"mottle"; while the same figure is given by Koch (19) as the thermal death point of "ringspot."

Chester (7) has demonstrated the existence of antigenic differences between three different strains of X designated potato mottle, potato ringspot, and masked potato mottle. It is possible, therefore, that serological methods may furnish the most exact means of distinguishing the various strains of virus X following further experimentation along these lines.

In the meantime it is clear that identification of X strains in general cannot be made solely on the basis of symptoms in tobacco or *Datura stramonium*. Such strains, for example, as X^G (29), X^D (3), and the Up-to-Date top-necrosis virus (9) are not distinguishable with any degree of certainty on these hosts. It is desirable, therefore, in attempting to identify strains of X, to use appropriate varieties of virus-free potato in conjunction with other solanaceous hosts.

The strains of X at present known to occur naturally in potato, and their characteristic reactions may be summarised as follows:—

X^B (Up-to-Date top-necrosis virus ((9) (14)).—Top-necrosis in President potato. Mottle in Arran Crest and Epicure. Mild mosaic in *Datura stramonium* and tobacco.

X^D ((3) (29)).—Severe foliar necrosis in President and certain other potato varieties. Top-necrosis in Arran Crest and Epicure. Mild mosaic in *Datura stramonium* and tobacco.

X^H (29).—Carried symptomlessly in President. Top-necrosis in Arran Crest and Epicure. Carried symptomlessly in *Datura stramonium* and tobacco. Mosaic and necrosis in *Capsicum annuum.*

Chester's (7) masked potato mottle is probably the same strain.

X^G (29) **or Simple Mosaic** (10).—Carried symptomlessly or very mild mottle in President potato. Top-necrosis in Arran Crest and Epicure. Mild mosaic in *Datura stramonium* and tobacco.

A slightly stronger form of X^G occurs, typified by the mosaic *ex* C described from this laboratory (10) and distinguishable only by the somewhat greater intensity of the symptoms in the non-potato hosts.

X^S (29).—Interveinal mosaic and slight foliar necrosis in President potato. Top-necrosis in Arran Crest and Epicure. Severe local and systemic necrosis and mosaic in *Datura stramonium* and tobacco.

X^N (29).—Severe foliar necrosis in President and other potato varieties, including a number which do not react similarly to X^D. Top-necrosis in Arran Crest and Epicure. Severe local and systemic necrosis and mosaic in *Datura stramonium* and tobacco.

The severe strain described in the present paper may be regarded as similar to X^N with the qualifications mentioned in the text.

Putnam's Yellow Mottle Virus (28).—Yellowish interveinal mottling on President potato. Indefinite mottling on tobacco. Characteristic yellow mosaic

in tomato. Thermal death point 73° C. The identity of Putnam's virus as a strain of X has not yet been generally accepted (29).

The above list is a slightly amended form of that drawn up by Salaman (29). Excepting the Yellow Mottle virus, all the strains included are related immunologically and serologically and have a thermal death point of 68°–70° C.

The fact that virus X appears not to be transmitted except by contact between diseased and healthy plants implies that infection with this virus, even in "field susceptible" varieties, should be easy to avoid in seed potato stocks propagated from X-free tubers. According to present knowledge one might reasonably assume that contamination of such stocks in the field could only arise from infected "volunteer" plants within the crop, or from perennial weeds if any such were hosts of virus X. Control of X in partially infected crops by rogueing of diseased plants should be effective, provided "contact" plants are also removed, whether or not these show disease symptoms or react positively to other tests for the presence of X. This precaution is important in view of the fact that X can reach the tubers of a potato plant without necessarily infecting the foliage to any appreciable extent. The best method of avoiding X naturally lies in the use of "field immune" varieties as advocated by Cockerham (13); but the number of such varieties which are desirable in other respects is as yet extremely small.

SUMMARY.

A disease occurring naturally in seed potato stocks and characterised by symptoms of severe mosaic and necrosis was found to be due to a strain of virus X.

The symptoms of the severe strain in 32 varieties of potato are described. In all except three, which reacted with top-necrosis, an initial phase of foliar necrosis and leaf drop was followed by severe mosaic and stunting in the current year of infection.

The disease in potato was characterised by a striking decrease in virulence, which took place usually in the 3rd year of infection of the clone, and occurred consistently in all varieties. The recovered plants contained a mild strain of X. Reasons are advanced for the theory that the recovery is due to mutation of the severe strain to a mild form.

In other solanaceous hosts symptoms consisted of local and systemic necrosis, severe mosaic, and stunting.

Of a large number of non-solanaceous plants inoculated with the severe strain, only one (*Lamium hybridum*) became systemically infected. *Veronica agrestis*, beetroot, and mangold, reacted with local lesions.

The physical properties and serological reactions of the severe strain are similar to those of other X strains. Plants infected with a mild strain are immune to infection with the severe strain by sap inoculation, but not by grafting.

The U.S.D.A. Seedling 41956 is immune to infection with the severe strain. In double-grafted plants over 2½ months old, severe X passed freely downwards through intermediate scions of 41956. Upward movement in plants of the same age was inhibited by the presence of the immune variety. The significance of these results in regard to movement of virus X within the potato plant is discussed.

In field experiments considerable spread of the severe strain resulted from contact between diseased and healthy plants of a tolerant variety. That no insect vector was involved was shown by the absence of infection in plants grown in proximity to, but not actually touching, disease sources.

Disease symptoms were rarely observed in the current year of infection in the field. The production on the same tuber of healthy and diseased shoots following field infection is discussed.

Plants of intolerant varieties of potato failed to become infected by contact with disease sources in the field.

The relation of the severe strain to Salaman's X^N is examined; and the problem of the identification of X strains is discussed.

EXPLANATION OF PLATES.

Plate 15.

Fig. 1.—Potato *var.* Majestic three weeks after inoculation with the severe strain of X.

Fig. 2.—Healthy plant *var.* President and plant infected by core-graft with severe X. The withered lower leaves of the diseased plant are obscured in the photograph.

Fig. 3.—Top of President plant from clone in 3rd year of infection, showing recovery in apical growth.

Plate 16.

Fig. 4.—Potato plant *var.* Kerr's Pink in 2nd year of infection wtih severe X, showing recovery in one shoot.

Fig. 5.—Leaf of tomato infected with severe X, showing characteristic inter-veinal necroses.

Fig. 6.—Field plants of President potato. Left, 4th year of infection; and right, 2nd year of infection of clone.

Plate 17.

Fig. 7.—*Datura stramonium* plants of same age, 13 days after inoculation from potato plants (*a*) in 3rd year of infection showing recovery (left), and (*b*) in 1st year of infection with the severe strain.

Fig. 8.—*Nicotiana glutinosa* 13 days after inoculation with the severe strain.

Fig. 9.—Tobacco plant (*var.* Orinoco) infected with the severe strain. The top of this plant was virus-free.

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FIG. 3

FIG. 1.

FIG. 1.

FIG. 5.

Photographs by G. H. McLEAN.

FIG. 7.

FIG. 8.

Photographs by G. H. McLEAN.

FIG. 9.

THE

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"STUDIES IN PEAT."

PART 13.

MONA WAX AND ITS CONSTITUENTS AS EMULSIFYING AGENTS.

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Chemistry Department, University College, Cork.

Price One Shilling.

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Chemistry Department, University College, Cork.

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In Part 12 of this series (1) it was shown that Mona wax, as extracted from Irish peat by such solvents as petrol-alcohol, trichlorethylene, etc., is capable of promoting the emulsification of water in certain hydrocarbon liquids. The water-in-oil (W/O) emulsions sponsored by the wax can contain a high percentage of water, and even when the concentration of the water has a value of 75 per cent. the emulsions are characterised by a good degree of permanence. The investigations described in the present paper will show *inter alia* that the various constituents of Mona wax have not equal values as emulsifying agents, and that the nature of the oil exercises an influence on the character of the emulsion formed when the wax is used as the agent. The effect of heat on wax-sponsored emulsions and the mechanism of the changes caused by increased temperature will also be described. In addition, the effects produced due to the addition of certain electrolytes to the water to be emulsified will be discussed.

Experimental.

Emulsions were in all cases prepared by the intermittent addition intermittent shaking method (1). Such a procedure permitted the comparison of the characteristics of different emulsions formed under approximately similar conditions. Tests for type were carried out as indicated (*loc. cit.*). Behaviour on ageing was judged by allowing the emulsions to stand in corked vessels at room temperature with, in most cases, gentle mixing at irregular intervals to redistribute creamed material. The contents of vessels were examined occasionally both by unaided vision and (after suitable dilution of representative samples with the oil) under the microscope, no cover-glass being used (1). The results of the investigations were as follows:—

Effectiveness of Mona wax as emulsifying agent according to the mode of its extraction from peat and its subsequent treatment (2). The following waxes were examined:—(A) a wax extracted from peat by trichlorethylene (97 per cent. soluble in "white spirit"), (B) a wax extracted from peat by trichlor-

ethylene, and rendered 100 per cent. soluble in "white spirit" by chemical treatment (2), (C) a wax extracted from peat by a mixed solvent (60 per cent. petroleum spirit and 40 per cent. isopropyl alcohol), and subsequently treated with 5 per cent. chlorosulphonic acid. In each case 0·2 g. of the wax was warmed with 20 ml. of liquid paraffin. In the case of wax (A) traces of the wax remained undissolved. In cases (B) and (C) the waxes dissolved completely. 60 ml. of distilled water was then emulsified in the resultant liquid, the temperature in each case being maintained at approximately 40° C. throughout the process. The following observations were made:—

Wax (A): Apart from the fact that the traces of insoluble wax-matter settled on the walls of the container, this wax gave the most viscous and qualitatively the best emulsion. Very little deterioration of the system occurred on three months' standing.

Wax (B): This wax gave an emulsion of good permanence and of almost paste-like consistency. On account of the absence of insoluble matter this wax was used in many of the investigations reported in this paper.

Wax (C): This wax promoted a good emulsion which, on standing for a period of three months, underwent only slight deterioration.

Characteristics of the emulsions formed in selected oils, using Mona wax (extracted from peat by trichlorethylene, and rendered 100 *per cent. soluble in hydrocarbon) as agent.* The oils chosen were liquid paraffin, benzene, toluene, xylene, and sunflower oil. In each case 0·1 g. of the wax was dissolved in 20 ml. of the warmed oil, and the distilled water added as already indicated, the temperature being kept at approximately 40° C. during the addition. The observations made were as follows:—

Liquid paraffin. A very viscous emulsion resulted from the use of 60 ml. of distilled water. The permanence of a water-in-liquid-paraffin system with the wax as emulsifying agent is being separately investigated by the technique of size-frequency analysis.

Benzene. A viscous emulsion was formed when 60 ml. of distilled water was added. The emulsion could initially be submitted to continuous rapid shaking without showing signs of undue deterioration. On ageing, however, deterioration of the system became evident. On allowing the emulsion to stand undisturbed for a period of two months, globules of water, large enough to be visible to the unaided eye, were observed in the system, and a bulk quantity of water had collected at the base of the containing vessel.

Toluene. The emulsification of 60 ml. of water in toluene resulted in a product which was qualitatively better than that obtained when benzene was used. Like the water-in-benzene emulsion, the emulsion in toluene could be submitted, when freshly made, to rapid continuous shaking without evident deterioration. On ageing the system behaved in a manner analogous to that observed in the water-in-benzene system. An emulsion formed under similar circumstances of 75 ml. of water in 20 ml. of toluene (which originally contained 0·1 g. of the wax) showed similar characteristics of stability and permanence,

but even after four months of standing no bulk quantity of water had collected at the bottom of the vessel. 0·4 g. of the wax was dissolved in 20 ml. of toluene, and an investigation made of the possibility of forming a very concentrated emulsion of water in toluene from the resulting solution, the mode of preparation and the temperature being as aforementioned. When 90 ml. of water had been added the emulsion broke.

Xylene. This, as a dispersion medium, was not nearly as effective as toluene or benzene. The emulsion given by 60 ml. of water was, by visual examination, of poor quality though fairly viscous. Continuous rapid shaking of the emulsion caused separation of some of the water in large drops. On ageing the system deteriorated rapidly.

Sunflower oil. This oil was chosen as a typical non-hydrocarbon liquid. The emulsion, which contained 60 ml. of dispersed water, contained fine globules when freshly prepared. It was of paste-like consistency. This emulsion was allowed to stand undisturbed for observation of its behaviour on ageing. It deteriorated more rapidly than the water-in-benzene and water-in-toluene emulsions, but less rapidly than the water-in-xylene emulsion.

Effect of heat on an emulsion of water in liquid paraffin. An emulsion was prepared containing 60 ml. of dispersed distilled water, 20 ml. of liquid paraffin, and 0·2 g. of wax (extracted from peat by trichlorethylene, and rendered 100 per cent. soluble in hydrocarbon; m. pt. of wax 75° C.) as emulsifying agent. The emulsion was allowed to stand overnight, and then divided into three parts. The separate portions were placed in test-tubes, and the vessels closed with corks. One of the containers was placed in a water-bath maintained at 90° C., another in a water-bath maintained at 80° C., and the third in a bath kept at 65° C. After 15 minutes it was observed that the phenomena of creaming and breaking were occurring simultaneously in the two systems kept at the higher temperatures, that of breaking occurring more rapidly in the system at 90° C. than in the system maintained at 80° C. After thirty minutes the volume of water which had collected as a bulk layer at the bottom of the test-tube was about 5 ml. from an emulsion of total volume 25 ml. in the case of the sample heated at 90° C., and somewhat less in the case of the sample at 80° C. The tubes and contents were then submitted to six single shakes, and the emulsion in each case reformed temporarily, only to break again rapidly to the same extent as before on resting in the appropriate water-bath. Again it was noted that the emulsion at 90° C. deteriorated more rapidly than that at 80° C. The emulsion in the 80° C. water-bath was then removed and cooled to 70° C., shaken six times, and maintained at the new temperature. The shaking again caused the emulsion to reform temporarily, but again water separated as a bulk layer on resting. In the case of the emulsion at 65° C. some breaking had occurred after one hour, but to a much less extent than at the higher temperatures.

An emulsion of 16 ml. of distilled water in 24 ml. of liquid paraffin, with 0·2 g. of the wax (of the same nature as that just mentioned) was prepared at

room temperature (16° C.), and its behaviour on ageing was observed at intervals by examination of the gently-mixed emulsion, suitably diluted, under the microscope. It was some weeks before the interfacial films became distinct.

Mechanism of the breaking due to heating. The permanence of water-in-liquid-paraffin emulsions sponsored by the wax is due to the envelopment of the water-globules by interfacial wax-films (1). When the wax itself dissolves in liquid paraffin it gives a yellow-brown colloidal solution. When a water-in-liquid-paraffin emulsion creams, the clear supernatant layer of liquid consists normally of a very dilute solution of the wax in liquid paraffin, as judged from the very faint yellow colour of the layer in question. When, however, the creaming was accompanied by breaking due to elevation of the temperature, the supernatant liquid was brown-yellow in colour, indicating that the breaking was associated with re-peptization (3) of the film-material by the liquid paraffin.

Effect of the presence of electrolytes in the water on the character of the emulsion formed. The following substances were dissolved in distilled water so as to give solutions of the concentrations indicated:—Sodium chloride, 0 1 per cent.; calcium chloride, 0·1 per cent.; magnesium chloride, 0·1 per cent.; magnesium sulphate 0·1 per cent.; aluminium sulphate, 0 1 per cent.; sodium oleate, 1 per cent.; hydrochloric acid, N/10; sodium hydroxide, N/10. Attempts were made to prepare, in each case, an emulsion containing 20 ml. of liquid paraffin, 0·2 g. of the wax (100 per cent. soluble in hydrocarbon), and 60 ml. of the solution of electrolyte, the temperature of preparation being 40° C. Results:—

With sodium chloride, calcium chloride, magnesium chloride, and magnesium sulphate solutions the products were more viscous than that obtained when distilled water was used, but the final emulsions were inferior to that produced when water alone was the internal phase. When these emulsions were allowed to rest undisturbed, deterioration set in. After two months of ageing, bulk-quantities of water or aqueous solution had collected at several points throughout the systems, but the unbroken parts of the emulsions, which were appreciable, still preserved a highly pasty consistency.

Aluminium sulphate. - The final product was again highly viscous and somewhat resembled that produced when a solution of sodium chloride was employed. During the later stages of the preparation of the emulsion (after 35 ml. of the electrolyte solution had already been emulsified), difficulty was experienced in bringing about emulsification of the solution, unemulsified liquid being visible in the system after the shakings. When the system had been standing for a period of two months, water or aqueous solution had collected at several points throughout the mass, but the pasty consistency of the remainder of the system was maintained.

Sodium oleate. The final product was a "multiple" system in which water or an aqueous solution was the primary external phase. The product was of low viscosity and creamed (upwards) rapidly.

Hydrochloric acid solution. After the emulsification of 50 ml. of the

aqueous solution, additional quantities of the dilute acid could not be emulsified by the method of preparation followed. The emulsified portion, in contact with the excess unemulsified liquid, remained reasonably permanent for four weeks.

Sodium hydroxide solution. When 50 ml. of the aqueous solution had been added the system, although viscous, was not of the same highly pasty consistency as that obtained, for example, when 0·1 per cent. sodium chloride solution was used. The remaining 10 ml. (50 ml. to 60 ml.) could not be emulsified well. Apart from the separation of this excess volume of liquid, the system underwent no evident gross change on standing undisturbed for a period of three weeks.

Individual constituents of the wax as emulsifying agents. The following constituents of the wax were tested for effectiveness as emulsifying agents: (A) the resinous matter which is soluble in cold alcohol (4 and 5), (B) the constituent removed from the wax (extracted from peat by trichlorethylene and rendered 100 per cent. soluble in hydrocarbon) by means of boiling alcohol (95 per cent.) which is precipitated from the alcoholic solution on cooling, (C) the matter in this wax which is not soluble in boiling alcohol, (D) the "total acids" in the wax (6). The following observations were made:—

(A) (resinous matter). 0·1 g. of the resin was dissolved in 20 ml. of liquid paraffin, and distilled water was added with shaking in the same manner as before, the temperature being maintained at 40° C. It was found impossible to emulsify more than 30 ml. of the water. The experiment was repeated using 0·2 g. and 0·25 g. of the resin in the same volume of liquid paraffin, the conditions of preparation being the same as before. With 0·2 g. of the resin about 30 ml. of water was emulsified, and with 0·25 g. of resin it was possible to emulsify 40 ml. of water. These emulsions were allowed to stand undisturbed in contact with the excess added water, for a period of three months. Rapid deterioration set in.

(B) (material dissolved by hot ethyl alcohol and precipitated on cooling the solution). 0·1 g. of this material, in 20 ml. of liquid paraffin, allowed 40 ml. of water to be emulsified, the temperature being maintained at 40° C. during the preparation. On allowing the system, with an excess of added water, to stand undisturbed for three months, it was found that about 15 ml. of the water originally emulsified still remained in the emulsified condition.

(C) (material insoluble in hot alcohol). This material was obtained by extracting the wax to exhaustion with 95 per cent. ethyl alcohol. The wax had to be refluxed with the alcohol a large number of times to remove completely the alcohol-soluble matter. 27·5 per cent. of the wax remained as insoluble pitch-like matter. This material was not peptized very readily by hot liquid paraffin. 0·1 g. of this substance, in 20 ml. of liquid paraffin, readily allowed the emulsification of 60 ml. of distilled water, the temperature of preparation being 40° C. The emulsion formed was of good quality and of paste-like consistency. An emulsion formed under similar circumstances of 60 ml. of

water in 20 ml. of liquid paraffin, with 0·2 g. of the alcohol-insoluble material as emulsifying agent, was so viscous when cold that it could not be poured from the container. The emulsions promoted by this constituent are being further investigated.

(D) (total acids). 0·1 g. of the total acids in 20 ml. of liquid paraffin gave a thick coarse emulsion when as much as 35 ml. of water had been added, the temperature of the preparation being 40° C. More than 35 ml. could not be emulsified under the conditions chosen. When the system (including the excess water) was allowed to stand undisturbed for a period of three months, only 10 ml. of the water originally emulsified had not separated in the bulk layer at the bottom of the container during this period of ageing.

Investigation of sodium salts of "total acids" and of resin acids as possible agents for the production of oil-in-water emulsions. With the exception of the "multiple" type of emulsion formed when the aqueous phase contained sodium oleate, all of the emulsions thus far described were of the water-in-oil type. Sodium and potassium soaps usually sponsor the oil-in-water type of emulsion. It was decided to determine whether the sodium salts of the total acids and of the resin acids had any value as emulsifying agents for the production of O/W emulsions.

A weighed quantity of the total acids (5) was dissolved in 95 per cent. ethyl alcohol, and an alcoholic solution of an estimated quantity of sodium hydroxide was added. The mixture was refluxed for 1 hour, and then allowed to cool. The soap which separated was filtered, washed with a little cold alcohol, then washed with ether, and finally dried. The soap was then dissolved in distilled water so as to give a solution which was estimated to have a concentration of approximately 0·01N. Of this solution 12 ml. was taken. For comparison purposes, 12 ml. of 0·01N aqueous sodium oleate was taken in another vessel. In either case 8 ml. of kerosene was added in 0·5 ml. quantities up to 3 ml., and then in 1 ml. quantities up to the 8 ml., with intermittent shaking at room temperature. The sodium salts of the acids appeared to have no value as emulsifying agents.

The product obtained when the resin (4 and 5) was (as far as possible) dissolved with the minimum amount of sodium hydroxide solution was compared under similar circumstances with the sodium compound of colophony. The colophony complex was a better emulsifying agent.

DISCUSSION OF RESULTS.

The fact that the nature of the oil exercises an influence on the character of the emulsion formed is not surprising. Numerous workers have commented on the phenomenon of specificity in emulsification processes (6). The effect of heat on the concentrated emulsion chosen suggests that, while emulsification is often facilitated by slight elevations of the temperature, the optimum temperature for the preparation of W/O emulsions promoted by the wax would

lie below 60° C. The effects observed when the aqueous phase contained electrolytes might have been due to physico-chemical influences of the electrolyte or, in certain cases, to the method of emulsion-preparation which was followed. Mere shaking of a system is, at best, an inferior method for the preparation of emulsions. In the cases where the aqueous phase contained dissolved inorganic salts, the resultant systems were already highly viscous before all of the aqueous solution had been added. The high viscosity already reached may have been a factor in determining the character of the final system. When sodium oleate was present in the aqueous phase, the "multiple" system obtained was due to the antagonistic influence of this soap (which, under the given conditions, favours the O/W type of emulsion). The superiority of the hot-alcohol-insoluble matter as an emulsifying agent is noteworthy.

Summary.

Mona wax (both untreated and treated so as to be 100 per cent. soluble in hydrocarbon) has been shown to be effective in sponsoring certain W/O emulsions.

The nature of the oil is a factor in determining the character of the emulsion formed. When the oil was toluene it was found possible, following the mode of preparation used, to prepare an emulsion containing more than 81 per cent. of dispersed water, using 0·4 g. of wax, per 20 ml. of toluene, as emulsifying agent.

The effect of heat on an emulsion of water in liquid paraffin with the wax as emulsifying agent has been studied, and it has been observed that at temperatures above 60° C. the breaking which occurs is associated with re-peptization of the film material by the liquid paraffin. It is considered that the optimum temperature for the preparation of such emulsions lies below 60° C.

The effect of the presence, in the water, of certain electrolytes has been examined. A solution of sodium oleate gave rise to a "multiple" system.

The components of the wax have different values as emulsifying agents. The constituent which gave the best result was the fraction insoluble in boiling 95 per cent. ethyl alcohol.

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A RAPID FERMENTATION METHOD FOR THE PRODUCTION OF CALCIUM CITRATE AND CALCIUM GLUCONATE FROM BEET MOLASSES.

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Introduction.

Since Wehmer's original patents in 1894 a great deal of research on the citric-acid-producing fungi has been carried out, resulting in the establishment in several countries of fermentation industries for the production of citric acid. The history of this fermentation is well reviewed by Wells and Herrick (1938). The details of the processes at present in operation are not available, but the method undoubtedly consists of shallow-pan fermentation of sucrose by *Aspergillus niger*, the process being completed in probably less than nine days. The mass of patent literature concerning this fermentation reveals little of actual plant operation.

The most recent work on the shallow-pan process to be published is that of Doelger and Prescott (1934). This method depends on the acidification of the medium to pH 1·6–2·2 with HCl, to suppress contaminating yeasts and bacteria. An unusual method of citric fermentation was advocated by Cahn (1935) in which a rapid formation of acid was obtained by using a relatively larger surface of mycelium. This was secured by growing the mould through a mass of bagasse or beet pulp impregnated with various sugar solutions.

The production of calcium gluconate by mould fermentation has been studied extensively by a number of workers in the U. S. Department of Agriculture. In general, it may be produced by the shallow-pan method or by using submerged mould growths under increased air pressure. The latter is one of the most interesting developments of fermentation technique in recent years. The method is described by Herrick, Hellbach, and May (1935), and has resulted in the conversion of a number of surface fermentation processes, with their attendant drawbacks, to a type of rapid deep fermentation.

The object of the work described in this paper was to develop a method of producing citric acid and calcium gluconate which might be practicable in this country under existing circumstances. Shortages of suitable carbohydrates had to be considered as well as scarcity of nitrates and phosphates for nutrient media. In working on this method the equipment which might be required for large-scale operation had also to be kept in mind.

Methods.

Fermentations, whether on liquid or absorbent media were carried out in tinned dishes, 12″ × 10″ × 2½″, which were double painted with cellulose lacquer, and covered by glass plates. When the fermentations were carried out on absorbent media, the mass was pressed out in a tincture press, and the pressed cake moistened and re-pressed twice. Citric acid was estimated by precipitation as calcium citrate, which was dried and weighed. Calcium gluconate was estimated by precipitation of the calcium as oxalate, which was washed and titrated by permanganate.

Experimental.

Doelger and Prescott's (1934) method was investigated by one of us (O.R.), and results in substantial agreement with those published were obtained. Layers of sugar solution and nutrients 2 5 cm. deep, acidified to pH 1 8–2·0, were inoculated with *Aspergillus niger.* Mats started to form on the third day and increased until the eighth or ninth day. Provided sporing did not take place before the fifth day, yields of 33–35 per cent. citric acid in nine days at 28° C. were obtained. The strength of the acid varied between 6 per cent. and 8 per cent. The mats could be used a second or third time to produce more citric acid if fresh sugar solution equal in strength (12 or 15 per cent.) to the original culture solution was run in. Oxalic acid was seldom produced by the first fermentation of a mat, but the second and third fermentations often produced small quantities. The acid yield and concentration of these fermentations was generally the same as in the primary fermentation. These laboratory experiments were reproduced with a total area of 40 sq. feet of mycelium, and similar results as to yield and strength were obtained. The main objections to the operation of this method under present circumstances in this country include: (1) the difficulty of securing metal pans; (2) the high cost and scarcity of the main raw material, sugar; (3) the length of the fermentation period; and (4) the liability of the *Aspergillus* mats to infection by a parasitic *Penicillium.* When this occurs the yield of citric acid is very low.

Cahn's Process.

An original method was described by Cahn (1935) in which molasses and other sugar-containing solutions are absorbed by beet pulp or bagasse, inoculated, and fermented for two to three days. The acid is recovered by diffusion, on the counter-current principle, from the chopped fermented mass. This method seemed to offer distinct advantages, and was accordingly investigated. Cahn, who used cane molasses, stated that beet molasses was toxic to his strain of *Aspergillus.* Bagasse not being available in Ireland at present, beet pulp impregnated with nutrient solution of the Doelger and Prescott (1935) formula was used. It was found very troublesome to get the dry beet

pulp saturated with solution, boiling for 30 min. being necessary. The growing mycelium then penetrated the soft tissues of the impregnated pulp, and the mass at the end of 48 or 60 hours had the consistency of soft cheese. The recovery of acid from this was difficult, nor could the high yields reported by Cahn be obtained. He may have used a strain of *Aspergillus* better adapted to the conditions of his method than those available to us.

THE SPHAGNUM METHOD.

Consideration of these results led to the trial of other absorbents available here. One of the most obvious of these is sphagnum moss, which is abundant and, by virtue of its peculiar leaf structure, an excellent retainer of moisture. Furthermore, it absorbs liquids by capillarity and not by imbibition, as in the case of beet pulp, and when used in the right proportion has a "channelled" texture through which air can penetrate to the germinating spores and mycelium. Finally, the fermented solution need not be diffused, but can be pressed out. The sphagnum from the bog is first pressed to remove surplus water, and then dried at a temperature of 80° C. to 100° C. This drying is important, as germination of the *Aspergillus* is retarded and yields are low when it is omitted. The moss is used in the proportion of 50 g. of air-dry sphagnum to 500–600 c.c. of solution, which is added hot. When cool, the mass is heavily inoculated with *Aspergillus* spores, and well mixed to distribute them. The spores germinate in 5–12 hours, and fermentation proceeds for 48 to 72 hours. The mycelium generally spores on the third day, but should be pressed out 48 hours after inoculation. The depth of saturated moss is between 1 and 2 inches, and the temperature of the fermentation chamber should be 25° C. If the depth is greater than 2 inches or the external temperature higher than 25° C., over-heating up to 42° C. tends to occur within the sphagnum. When this happens the yields are always low.

Other absorbents tested were apple pomace, malt combings, and softwood sawdust. The first two were unsatisfactory, their low water absorbing powers necessitating the use of relatively large quantities. In the case of apple pomace, the mycelium tended to grow on the surface of the mass. With malt combings the final condition resembled the cheese-like mass obtained when beet pulp was used; also yields of citric acid were very low. Sawdust used in the proportion of 120 g. per 600 c.c. of solution was quite satisfactory, giving yields comparable to those obtained on sphagnum.

Three different fermentable solutions were used in this work, the first being the Doelger and Prescott (1934) solution—

Sucrose	150	grams per litre acidified to pH 2·0 with HCl.
NH_4NO_3	2·3	
KH_2PO_4	1·0	
$MgSO_4$	0·25	

On a two-day fermentation the average yield of citric acid in three experiments was only 15 per cent. of the sugar taken, and a quantity of oxalic acid was formed. Allowing the fermentation to proceed for 3 days did not improve the yield. When the sucrose concentration was raised to 20 per cent. the yield in two days was increased to 18 per cent., and the tendency to form oxalic acid was markedly reduced. Different strains of *Aspergillus* were then tried, but did not effect any improvement. The only apparent advantages in using this formula with sphagnum, instead of in shallow pans, were the freedom from infection and the shorter fermentation time. These advantages were offset by the low yields of acid.

Attention was then turned to molasses, which is a cheap source of sucrose and contains sufficient minerals and nitrogenous materials for the growth of *Aspergillus*. While Cahn (1935) has reported beet molasses as being toxic to his strain of *Aspergillus*, it was felt that it might be possible to overcome this toxicity. Chattergee (1942) describes a shallow-pan method of fermenting molasses—presumably cane molasses—below pH 3·5, using aluminium pans. Our first experiments with beet molasses at different concentrations up to 40 per cent., and at pH 2·0, 3·0, and 5·0, in liquid form in shallow pans were always unsuccessful. Spore germination and growth of the mats was very slow, while infection was heavy. Small amounts of citric acid were obtained by growing the mould in conical flasks containing 200 c.c. of sterile 25 per cent. molasses, yielding 2·91 g. of calcium citrate in 10 days, and 4·62 g. in 14 days. However, when sphagnum or sawdust was impregnated with dilute beet molasses, rapid growth took place, in marked contrast to the poor growth on liquid molasses media. Acidification to pH 2·0 practically stopped growth, consequently the diluted molasses was used at its natural pH (7·2).

The fermentation medium in these experiments was 200 g. molasses brought to boiling point with 450 c.c. water, poured on to 50 g. dry sphagnum, inoculated when cool and fermented for two days. The pressings were neutralised with calcium carbonate, filtered, and boiled to precipitate calcium citrate. The yields of these experiments were 10–12 g. of citrate per 200 g. of molasses. Adding NH_4NO_3 and KH_2PO_4, together or separately, to the molasses mashes did not improve the yields. Though the results were poor, these experiments did show that beet molasses could be fermented in two days to produce some citric acid.

The next change in the method was influenced by the following considerations:—

1. Analyses figures of Irish beet molasses show a very high ratio of sodium and potassium ions to calcium, and it was thought that increasing the calcium content of our molasses mashes might antagonise the possibly deleterious effects of this high concentration of sodium and potassium ions.

2. Moyer *et al.* (1937) have described a method of obtaining calcium gluconate by fermenting sugar solutions containing calcium carbonate

with *A. niger* in rotating drums under air pressure. This method gave high yields with a short fermentation period. In view of the short fermentation period with sphagnum molasses for citric acid it was thought that calcium gluconate might also be produced by the sphagnum method without the necessity for the elaborate pressure drums of Moyer.

3. The presence of calcium carbonate in the fermentation mixture has been claimed by some workers (Wehmer, 1893, Chrzaszcz and Peyros 1935) to increase the yield of citric acid, but in the shallow-pan method its use naturally favours contamination.

A preliminary experiment using a sugar solution of the following formula was set up:—

Sucrose	- 100 g.	Sphagnum moss	- 50 g.
NH_4NO_3	- 1·5 g.	$CaCO_3$	- 25 g.
KH_2PO_4	- 0·15 g.	Water	to 600 c.c.
$MgSO_4 7H_2O$	- 0·12 g.		

Growth of the *Aspergillus* was quite vigorous, and after three days the mass was pressed. The pressings were filtered from unused carbonate, and, on boiling, a precipitate of 9·65 g. of calcium citrate was obtained. When the citrate was filtered off, the filtrate was evaporated to a syrup, and, on standing, a copious precipitate of calcium gluconate was obtained.

As a result of some 70 experiments using molasses the following formula was found to give the most satisfactory yields:—

Molasses - - -	200 g.
Sphagnum - - -	50 g.
Calcium carbonate - -	40 g.
Water - -	to 600 c.c.

Growth on this medium at a temperature of 25° C. was vigorous, and when the fermentations were pressed, filtered, and boiled, copious precipitates of calcium citrate were obtained. The yields varied somewhat with individual pans, but in most cases ranged between 25 g. and 30 g. of calcium citrate per pan in two days. Increasing the time to three days did not improve the yield of citrate, neither did the addition of traces of $ZnSO_4$ and $Fe_2(SO_4)_3$. Increasing the molasses to 300 g. per pan gave relatively lower yields. A higher temperature than 25° C. always resulted in overheating of the fermenting moss; after the spores had germinated, the internal temperature would rise to 42° C. some 24 hours after inoculation. Overheated pans always gave negligible quantities of citrate. Using the impregnated sphagnum at a greater depth than 1·5 inches in an attempt to increase the output per pan also resulted in overheating, even at an air temperature of 25° C. Whether this heating was due to respiration of the mycelium or thermophilic bacteria was not investigated. The calcium citrate obtained from molasses was a pale-

grey powder, which gave a dilute solution of light-brown citric acid on treatment with sulphuric acid. This dilute citric acid was decolorised with charcoal, and on concentration crystallised readily to a white or very pale-yellow product.

The fermented molasses from which the citrate had been filtered was evaporated down to 200 c.c., and on standing calcium gluconate crystallised out. This gluconate could be freed from molasses residues by pressing in cloth, or by washing with small amounts of water. This procedure resulted in a pale-brown product which could be further purified by treatment with charcoal and re-crystallising from water to give white calcium gluconate.

The yields of gluconate—which was produced simultaneously with the citrate—varied with individual pans, but in general lay within the range of 50 g. to 65 g. of calcium gluconate per 200 g. molasses in a two-day fermentation.

Re-examination of the three-day molasses-sphagnum fermentations without calcium carbonate showed that much less gluconate was produced, as might be expected. Without calcium carbonate, 200 g. molasses yielded only 20–25 g. of gluconate on neutralisation. Some workers cited by Prescott and Dunn (1940) have claimed that gluconic acid is a stage in the formation of citric acid. When sphagnum was impregnated with a solution of 100 g. calcium gluconate in 600 c.c. water, plus a little molasses to supply nutrients, the mycelium developed well, but no citrate was produced. In this method it seems that calcium gluconate will not ferment to citric acid.

The results of all these experiments may be summed up in the following table:—

Medium.	Method.	Time. Days.	Ca. Citrate. Grams.	Ca. Gluconate. Grams.
100 g. Sucrose + Nutrient salts (1)	Shallow Pan	9	48·3	0
100 g. Sucrose + Nutrient salts (1)	Sphagnum Moss	2	27·1	0
200 g. Molasses. Water to 600 c.c.	Sphagnum Moss or Sawdust	2–3	11	20–25
200 g. Molasses. 40 g. $CaCO_3$. Water to 600 c.c.	Sphagnum Moss or Sawdust	2	25–30	40–45

(1) NH_4NO_3 1·15 g., KH_2PO_4 0·5 g., $MgSO_4$ 0·12 g., Water 500 c.c.

Discussion.

In emergency conditions the sphagnum method offers many advantages over the shallow-pan process. Large areas of acid-proof metal pans and quantities of NH_4NO_3 and KH_2PO_4 are not required. Trays built of asbestos

slate screwed to wooden battens have given satisfactory yields in the laboratory. The rapid fermentation rate prevents the infection by other fungi, which was found to be very troublesome in the 9-day process. Bacterial contamination might be expected from the working conditions of the method, but it was never noticed; this may be due to the secretion of antibiotics by *A. niger.*

Although the yield of citric acid on the sugar in molasses is lower than in the shallow-pan method, this is offset by the cheapness of molasses, and the rapidity and certainty of the fermentation. The value of the calcium gluconate produced simultaneously must also be considered. The total yield of citric acid plus gluconate is in the neighbourhood of 60–65 per cent. of the sugar present in the molasses.

Other minor advantages of this method are: (*a*) that on a large scale it avoids the difficulties of handling large volumes of liquid at a shallow depth, and (*b*) the troublesome neutralisation of dilute citric acid with calcium carbonate takes place during the fermentation as the acid is formed.

The process differs from Cahn's method in several important respects. The fermentation products are pressed out, not diffused. Beet molasses, which Cahn found to be toxic, is rendered usable by the addition of calcium carbonate. Calcium gluconate is produced along with calcium citrate. Since the effect of calcium carbonate on the gluconic acid fermentation of *A. niger* is well known (Prescott-Dunn, 1940, p. 380), it is not surprising that the yield of gluconate is improved by its addition.

It is not generally considered necessary to add calcium carbonate in the citric acid fermentation by the pan method, as its use favours contamination. Nevertheless the earlier workers did use calcium carbonate to increase the yield of citric acid. According to May and Herrick (1930) the infections favoured by the presence of calcium carbonate during a nine-day fermentation caused the process to be abandoned. Recent workers, as quoted earlier in this paper, have mainly tended to use sucrose media of very low pH with the surface method. Although a good deal of the conflicting data of various workers on citric acid may be due to the idiosyncrasies of different strains of *A. niger*, there is no doubt that the addition of calcium carbonate to the molasses solutions in this method nearly trebled the yield of calcium citrate. Further experiments using other strains of *Aspergillus* might result in increasing the ratio of citrate to gluconate.

SUMMARY.

1. The shallow-pan process and Cahn's method have been investigated. For various reasons neither method is practicable under present conditions.
2. A method of fermenting beet molasses with *Aspergillus niger* on a base of sphagnum moss or sawdust is described.
3. The fermentation time is two days, calcium citrate and calcium gluconate being produced simultaneously.

We wish to record our thanks to Professor Doyle for his continued interest in the progress of this work, and to the Research Department of the Irish Sugar Co., Ltd., who kindly supplied us with data on beet molasses. The process described in this paper has been allocated a Provisional Patent 325/43.

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